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(54) Title: **INDUCIBLE SCREEN FOR DRUG DISCOVERY**

(57) Abstract: A method for identifying a polynucleotide, the expression of which is modulated in the presence of nitric oxide (NO), which method comprises: (i) providing an mRNA or cDNA population from cells which contain a polynucleotide construct, which construct comprises: (a) a promoter operably linked to a coding sequence, wherein the promoter is responsive to ecdysone or an analog thereof and the coding sequence encodes a nitric oxide synthase (NOS) or a functional variant thereof; or (b) a promoter operably linked to one or more tetracycline operator site sequences and a coding sequence in that order, wherein the coding sequence encodes a nitric oxide synthase (NOS) or a functional variant thereof; (ii) providing an mRNA or cDNA population from cells as defined in step (i), said cells having been contacted with ecdysone or an analog thereof; and (iii) comparing the populations of steps (i) and (ii), thereby to determine which polynucleotides show modulated expression in the presence of NO.

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INDUCIBLE SCREEN FOR DRUG DISCOVERY

5 **Field of the Invention**

This invention relates to methods for identifying genes which are differentially expressed in the presence of nitric oxide. It also relates to cancer therapy.

10 **Background to the Invention**

Nitric oxide (NO) is a pleiotropic signal molecule which has been identified as a mediator for a wide range of physiological and pathophysiological events. The diverse cellular signalling properties of NO are in part, due to redox-sensitive interactions with metal and thiol containing proteins. One major downstream target
15 is the enzyme soluble guanylyl cyclase (sGC). NO binding to the heme domain of sGC results in a 200-400 fold increase in enzymatic activity, leading to an increase in the concentration of the intracellular messenger molecule cGMP.

It has also been shown that NO can mediate some of its biological effects through mechanisms involving the transcriptional regulation of a number of
20 molecules including the p21^{WAF1} cyclin dependent kinase inhibitor and, in the presence of calcium, c-fos and c-jun. In addition, NO can also increase p53 protein concentration. This results in an increase in p53 stability and is likely to have effects on the transcription of p53-regulated genes. NO can also transcriptionally regulate the expression of the vascular endothelial growth factor, VEGF.

25 We are interested in the effects of NO on gene expression, and particularly in how an analysis of this process may help uncover some of the roles of NO in disease and health. This approach has enormous potential for the identification of novel genes in a variety of disease states as these NO regulated genes are likely to constitute novel targets for drug development strategies.

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Summary of the Invention

In order to examine the role of NO on gene expression we have used a differential display protocol, involving the differential hybridization of mRNA-derived probes to normalized cDNA arrays.

5 Using this approach, we have shown that NO can upregulate DNA-dependent protein kinase (DNA-PK) activity by increasing transcription of the DNA-dependent protein kinase (DNA-PKcs) gene.

To test the biological significance of this upregulation we exposed cells to high doses of DNA damaging agents, such as NO donors, bleomycin, adriamycin, 10 cisplatin, UV-C irradiation and X-ray irradiation. We have shown that NO generating cells, with increased levels of DNA-PK, are protected against UV-C, X-ray irradiation, bleomycin, adriamycin, cisplatin, as well as to high concentrations of NO donors. Remarkably, however, a NOS inhibitor or a DNA-PK inhibitor can abolish this protection.

15 These results are highly significant in the context of cancer therapy. The finding that human cancer cells express NOS indicates that NO may play a pathophysiological role in promoting tumor growth and in protecting tumors from agents that cause DNA damage. NO production in cancer cells may thus confer resistance to chemotherapeutic drugs, such as bleomycin and cisplatin, and to 20 radiotherapy on those cells.

Acquired drug resistance is a major problem in cancer treatment. Our findings suggest that NO production may underlie resistance to some widely used cancer therapies. These findings open up a totally new strategy for cancer therapy, suggesting that administration of DNA-damaging drugs in combination with 25 inhibitors of NOS or DNA-PK can sensitize NO-producing tumor cells that would otherwise be resistant to DNA-damaging drugs or radiation.

According to the present invention there is thus provided a method for identifying a polynucleotide, the expression of which is modulated in the presence of 30 nitric oxide (NO), which method comprises:

- (i) providing an mRNA or cDNA population from cells which contain a

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polynucleotide construct, which construct comprises:

- (a) a promoter operably linked to a coding sequence, wherein the promoter is responsive to ecdysone or an analog thereof and the coding sequence encodes a nitric oxide synthase (NOS) or a functional variant thereof; or
- (b) a promoter operably linked to one or more tetracycline operator site sequences and a coding sequence in that order, wherein the coding sequence encodes a nitric oxide synthase (NOS) or a functional variant thereof;

- (ii) providing an mRNA or cDNA population from cells as defined in step (i), said cells having been contacted with ecdysone or an analog thereof; and
- (iii) comparing the populations of steps (i) and (ii), thereby to determine which polynucleotides show modulated expression in the presence of NO.

The invention also provides:

- use of a polynucleotide identified by a method for identifying a polynucleotide, the expression of which is modulated in the presence of nitric oxide (NO), in a method for identifying an inhibitor or stimulator of transcription and/or translation of the polynucleotide and/or activity of the polypeptide encoded by that polynucleotide;
- a method for identifying:
 - (i) an inhibitor or stimulator of transcription and/or translation of a polynucleotide identified by a method for identifying a polynucleotide, the expression of which is modulated in the presence of nitric oxide (NO); and/or
 - (ii) an inhibitor or stimulator of activity of a polypeptide encoded by a said polynucleotide,

which method comprises determining whether a test substance can inhibit or stimulate transcription and/or translation of the polynucleotide and/or activity

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of a polypeptide encoded by a said polynucleotide.

- an inhibitor or stimulator identified by a method for identifying:
 - (i) an inhibitor or stimulator of transcription and/or translation of a polynucleotide identified by a method for identifying a polynucleotide, the expression of which is modulated in the presence of nitric oxide (NO); and/or
 - (ii) an inhibitor or stimulator of activity of a polypeptide encoded by a said polynucleotide.
- an inhibitor or stimulator of the invention for use in a method of treatment of the human or animal body by therapy;
- a polynucleotide construct comprising:
 - (a) a promoter operably linked to a coding sequence, wherein the promoter is responsive to ecdysone or an analog thereof and the coding sequence encodes a nitric oxide synthase (NOS) or a functional variant thereof; or
 - (b) a promoter operably linked to one or more tetracycline operator site sequences and a coding sequence in that order, wherein the coding sequence encodes a nitric oxide synthase (NOS) or a functional variant thereof;
- a vector which incorporates a polynucleotide construct of the invention;
- a cell which harbours a polynucleotide construct of the invention or a vector of the invention;
- products containing an NOS inhibitor and a DNA damaging agent as a combined preparation for simultaneous, separate or sequential use in the treatment of cancer;
- products containing a DNA repair enzyme inhibitor and a DNA damaging agent as a combined preparation for simultaneous, separate or sequential use in the treatment of cancer;
- use of an NOS inhibitor in the manufacture of a medicament for use with a DNA damaging agent in the treatment of cancer;
- use of a DNA repair enzyme inhibitor in the manufacture of a medicament for

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- use with a DNA damaging agent in the treatment of cancer,
- a method of treating a host suffering from a cancer, which method comprises administering to the host therapeutically effective amounts of an NOS inhibitor and a DNA damaging agent;
 - 5 - a method of treating a host suffering from a cancer, which method comprises administering to the host therapeutically effective amounts of a DNA repair enzyme inhibitor and a DNA damaging agent;
 - use of an NOS inhibitor in the manufacture of a medicament for use in the treatment of retroviral infection;
 - 10 - use of an NOS inhibitor in the manufacture of a medicament for use with a PI 3-kinase like kinase inhibitor in the treatment of retroviral infection;
 - products containing an NOS inhibitor and a PI 3-kinase like kinase inhibitor as a combined preparation for simultaneous, separate or sequential use in the treatment of retroviral infection;
 - 15 - a method of treating a host suffering from retroviral infection, which method comprises administering to the host a therapeutically effective amount of an NOS inhibitor; and
 - a method of treating a host suffering from retroviral infection, which method comprises administering to the host therapeutically effective amounts of an
 - 20 NOS inhibitor and a PI-3 kinase like kinase inhibitor.

Brief description of the Drawings

Figure 1(a) shows the plasmid map for pIND-hiNOS-f (human iNOS); Figure 1(b) shows the plasmid map for pIND-hnNOS-f (human nNOS); and Figure 1 (c) shows the plasmid map for p-IND-heNOS-f (human eNOS). Figure 1(d) shows the plasmid map of pTet-hiNOS-f (human iNOS).

Figure 2(a) shows generation of NO by EcR293 clone 11, following treatment with muristerone A. EcR293 clone 11 cells were grown with varying concentrations of muristerone A and at different time intervals supernatants were taken and the Griess reaction was used to measure the nitrite concentration. Figure 2(b) shows

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Northern and Western blots of carried out on extracts from cells treated with either 1 μ M or 10 μ M muristerone A. Extracts were also taken from cells grown in the absence of muristerone A. For Northern blots, filters were probed with a human iNOS cDNA and hybridisation with human β -actin was used as a loading control.

5 For Western blots, filters were probed with a polyclonal antibody raised against the 7 C-terminal residues of human iNOS: Cyc-Arg-Nle-Orn- (Ser-Leu-Glu-Met-Ser-Ala-Leu). The filters were subsequently stripped and re-probed with an anti- human α -tubulin antibody as a loading control.

10 Figure 3 shows generation of NO in a panel of cell lines transfected with pTet-hiNOS-f. Cells were treated with 1 μ g/ml tetracycline for 24h and NOS activity was assessed by assaying for accumulated nitrite using the Griess reaction.

15 Figure 4 shows Northern blot analysis for DNA-PKcs. PolyA⁺ mRNA was extracted from cells grown in the absence of NO (none) and in its presence following treatment with 1 μ M and 10 μ M muristerone A, and either with or without the NOS inhibitor L-NIO (20 μ M) for 24 hr. Figure 4(a) shows that DNA-PKcs mRNA is significantly increased in the presence of NO. Figure 4(b) shows that the level of DNA-PKcs is reduced by addition of the NOS inhibitor L-NIO (20 μ M).

20 The DNA-PK signal is expressed as a percentage (\pm S.D) of the β -actin signal as an average from three separate experiments.

25 Figure 5 shows Western blot analysis of DNA-PKcs expression. Figure 5(a), total cell extracts were separated by electrophoresis and immunoblotted with anti-DNA-PKcs antibody. The order of the tracks are: untreated (none), treated with 1 μ M muristerone A, 5 μ M muristerone A, 10 μ M muristerone A and 10 μ M muristerone A in the presence of 20 μ M L-NIO. Figure 5(b) shows nuclear lysates. The order of the tracks are: untreated cells (none) and cells treated with 1 μ M and 10 μ M muristerone A.

30 The filters were stripped and reprobed with an antibody against Ku-80 as a control for equal loading (Figure 5(a) and 5(b), bottom panels).

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Figure 6 shows DNA-PK pulldown peptide assays. Figure 6(a) shows samples prepared from control EcR293 clone 11 cells, and cells expressing NO after treatment with 1 μ M or 10 μ M muristerone A in the presence of 20 μ M L-NIO. Peptides derived from wild type and mutant p53 peptide were used as substrate.

5 Figure 6(b) shows DNA-PK activity in nuclear extracts prepared from cells treated with muristerone A as described above. Averages and S.D values from three independent experiments are shown.

Figure 7 shows protection of NO-generating cells from X-ray irradiation.

10 Survival rates of EcR293 clone-11 cells two weeks after X-ray irradiation are shown. Open squares, untreated cells; diamonds, control untransfected cells treated with 10 μ M muristerone A; closed squares, iNOS-expressing cells treated with 10 μ M muristerone A. Values are means \pm s.d from two separate experiments, each with two replications.

15 Figure 8 shows protection of muristerone A induced cells from DNA damaging agents. Figure 8(a) shows the viability of muristerone (10 μ M) induced EcR293 clone 11 cells following treatment with SNAP, bleomycin, adriamycin and cisplatin as judged by the trypan blue assay after a 72hr treatment. Figure 8(b) shows

20 cisplatin resistance of muristerone A induced EcR293 clone 11 cells and sensitization to 20 μ M wortmannin. Figure 8(c) shows dose-dependent death of EcR293 clone 11 cells following UV-C irradiation. Figure 8(d) shows UV-C irradiation of human EcR293 clone 11 cells: UV-C irradiation of EcR293 clone 11 cells (120mJ/cm²) following treatment with 1 μ M, 10 μ M and 10 μ M muristerone A in the presence of

25 20 μ M L-NIO, 10 μ M muristerone A in the presence of 20 μ M wortmannin (WM). The cells were incubated for a further 24hr and cell viability was judged by trypan blue exclusion and lactate dehydrogenase activity assays. The last column represents the data for cells cultured in 10 μ M muristerone A and treated with 20 μ M wortmannin in the absence of UV-C irradiation. Comparisons were made between

30 means \pm SD(n=4) of uninduced cells and cells treated with 10 μ M muristerone A. **, P<0.01.

Detailed Description of the Invention

Constructs, Vectors and Cells

5 Steroid hormones are small hydrophobic molecules that can diffuse through the plasma membrane of cells where they can bind reversibly to specific steroid-hormone-receptor proteins in the cytoplasm or nucleus. The binding of hormone activates the receptor, enabling it to bind with high affinity to specific DNA sequences that act as transcriptional enhancers. This binding increases the level of
10 transcription from certain nearby genes.

 A pulse of the insect steroid hormone ecdysone triggers metamorphosis in *Drosophila melanogaster*, showing effects such as chromosomal puffing within minutes of hormone addition. Mediating this response is the functional ecdysone receptor, which is a heterodimer of the ecdysone receptor (EcR) and the product of
15 the ultraspiracle gene (USP).

 Insect hormone responsiveness can be recreated in cultured mammalian cells by cotransfection of a cell with a functional ecdysone receptor (a heterodimer of EcR and USP) and an ecdysone responsive construct and treatment of the cell with ecdysone or an analog thereof.

20 A tetracycline responsive system can be created in cultured mammalian cells by cotransfection of a cell with a plasmid encoding a tetracycline repressor protein (tetR) and a plasmid containing a tetracycline responsive element linked to a promoter. The promoter sequence is used to drive heterologous gene expression. The tetracycline responsive element comprises particular DNA sequences called
25 tetracycline operator sites, which can bind a homodimer of tetR. If those sequences are positioned between a promoter and a coding sequence in a construct, the presence of tetR bound to a tetracycline operator site will prevent the promoter driving expression of the coding sequence. However, when tetracycline is added to cells the tetracycline binds to tetR homodimers leading to a conformational change in tetR,
30 such that it is unable to bind a tetracycline operator site. The tetR:tetracycline complex dissociates from the Tet operator site and allows the promoter to drive

expression of the coding sequence.

(i) Constructs

The invention provides polynucleotide constructs which are responsive to ecdysone or an analog thereof. The invention also provides polynucleotide constructs which are responsive to tetracycline or an analog thereof.

The ecdysonse responsive constructs comprise a promoter operably linked to a coding sequence, wherein the promoter is responsive to ecdysone or an analog thereof and the coding sequence codes for a NOS or a functional variant or fragment thereof.

The tetracycline responsive constructs comprise a promoter operably linked to one or more tetracycline operator site sequences and a coding sequence in that order, wherein the coding sequence codes for a NOS or a functional variant or fragment thereof.

The constructs may comprise DNA or RNA. They may also include within them synthetic or modified nucleotides. A number of different types of modification to polynucleotides are known in the art. These include methylphosphonate and phosphorothioate backbones, addition of acridine or polylysine chains at the 3' and/or 5' ends of the molecule. For the purposes of the invention, it is to be understood that the constructs described herein may be modified by any method available in the art. Such modifications may be carried out in order to enhance the *in vivo* activity or lifespan of constructs of the invention. Constructs of the invention may be produced recombinantly, synthetically, or by any means available to those of skill in the art.

The term "operably linked" refers to a juxtaposition wherein the components described are in a relationship permitting them to function in their intended manner. Thus, a regulatory sequence, such as a promoter, "operably linked" to a coding sequence is positioned in such a way that expression of the coding sequence is achieved under conditions compatible with the regulatory sequence.

A promoter for use in an ecdysone-responsive construct of the invention may be any promoter which can drive the transcription of a coding sequence to which it is operably linked in the presence of the steroid hormone ecdysone or an analog thereof.

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The promoter may be a naturally occurring promoter from a *Drosophila melanogaster* or other insect ecdysone-responsive gene. Alternatively, the promoter may be a non-naturally occurring promoter. A non-naturally occurring promoter may be used which comprises a minimal promoter and an ecdysone-responsive element (EcRE). An EcRE is a nucleotide sequence to which a functional ecdysone receptor can bind in the presence of ecdysone. Suitable minimal promoters include the minimal heat shock promoter.

An ecdysone-responsive promoter may comprise more than one EcRE, for example 2 to 10 elements or more preferably 4 to 6 elements. The sequence of an EcRE will depend on the exact functional ecdysone receptor used. If a modified functional ecdysone receptor is used (see below) it may be appropriate to use a modified EcRE (see No *et al.*, *Proc. Natl. Acad. Sci. USA*, 93: 3346-3351). The EcRE(s) and minimal promoter sequences do not have to be immediately adjacent. Because EcREs function as transcriptional enhancers, they can be placed some distance upstream, for example from 1, 10 or 25 nucleotides to 30, 40, 50, 100, 500 or 1000kb upstream of a minimal promoter. EcREs could even be placed further than 1kb upstream of a minimal promoter. Generally, if multiple copies of an EcRE are used, the multiple copies will be arranged in an array, one after the other.

Constructs of the invention may be responsive to ecdysone [(2 β , 3 β , 5 β , 22R)-2,3,14,22,25-pentahydroxycholest-7-en-6-one] or an analog thereof. Suitable analogs of ecdysone for use in the invention include muristerone A [2 β , 3 β , 5 α , 11 α , 14, 20, 22-heptahydroxy-5 β , 7-cholesten-6-one] or ponasterone A [(2 β , 3 β , 5 β , 22R)-2, 3, 14, 20, 22, 25-pentahydroxycholest-7-en-6-one] and GSTM-E (Invitrogen, San Diego, CA; see also Dhadialla *et al.*, 1998, *Ann. Rev. Entomol.* 43: 545-569).

A promoter for use in an tetracycline-responsive construct of the invention may be any promoter which can drive the transcription of a coding sequence to which it is operably linked in the presence of the antibiotic tetracycline or an analog thereof.

Generally, the choice of promoter will depend on the host cell to be used for expression of the coding sequence. Typically, expression in mammalian cells, for example human cells will be required and thus a mammalian promoter will be

preferred. Mammalian promoters, such as β -actin promoters, may be used. Tissue-specific promoters may be used. Viral promoters may also be used, for example the Moloney murine leukaemia virus long terminal repeat (MMLV LTR), the rous sarcoma virus (RSV) LTR promoter, the SV40 promoter, the human cytomegalovirus (CMV) IE promoter, adenovirus, HSV promoters (such as the HSV IE promoters), or HPV promoters, particularly the HPV upstream regulatory region (URR). Viral promoters are readily available in the art. Constitutive promoters, for example the CMV promoter, are preferred.

Tetracycline-responsive constructs comprise one or more tetracycline operator site (TetO_2) sequences, situated between the promoter and coding sequence. For example, two, three, four or even up to ten TetO_2 sequences may be used. Typically, if more than one TetO_2 site is used those sites will be arranged in the form of an array. However, other intervening nucleotide sequences may be situated between individual TetO_2 sites. For example one, two, three, four, five, up to ten or up to 15 nucleotides may intervene between any two TetO_2 sites.

The TetO_2 sequence is 5'-TCCCTATCAGTGATAGAGA-3' (Hillen and Berens, 1994, *Annu. Rev. Microbiol.* **48**, 345-369; Hillen *et al.*, 1983, *J. Mol. Biol.* **169**, 707-721) or a functional variant thereof. The TetO_2 sequence or a functional variant thereof is capable of being bound by a homodimer of tetR or a functional variant thereof.

A functional variant of the TetO_2 sequence is a sequence which is similar to that of the TetO_2 sequence and which remains capable of binding a homodimer of tetR or a functional variant thereof. The affinity of tetR for the TetO_2 sequence is $K_B = 2 \times 10^{11} \text{ M}^{-1}$ (as measured under physiological conditions), where K_B is the binding constant (Hillen and Berens, 1994, *supra*). The binding affinity of tetR for a functional variant of the TetO_2 sequence may be substantially the same as that of tetR for the TetO_2 sequence. Alternatively, tetR may have a binding affinity for a functional variant of the TetO_2 sequence which is greater or less than that of tetR for the TetO_2 sequence. For example, the affinity of tetR for a functional variant of the TetO_2 sequence may be from $K_B = 2 \times 10^9 \text{ M}^{-1}$ to $2 \times 10^{13} \text{ M}^{-1}$ or more preferably from $2 \times 10^{11} \text{ M}^{-1}$ to $2 \times 10^{12} \text{ M}^{-1}$.

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A functional variant of TetO₂ typically comprises a sequence substantially similar to that of the TetO₂ sequence. Thus, a functional variant of TetO₂ will generally have at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 98% or at least 99% sequence identity to the TetO₂ sequence, calculated over the full length of those sequences.

A functional variant of the TetO₂ sequence may be a modified version of that sequence obtained by, for example, nucleotide substitution or deletion. Up to 1, up to 2, up to 3, up to 4, up to 5, up to 6 or more nucleotide substitutions or deletions or combinations thereof may be made to the TetO₂ sequence to produce a functional variant of that sequence.

Constructs of the invention may be responsive to the antibiotic tetracycline or an analog thereof. Tetracycline binds to tetR homodimers, such that the tetR:tetracycline complex dissociates from the TetO₂ sequence. The association constant of tetracycline to tetR is $3 \times 10^9 \text{ M}^{-1}$. Preferred analogs of tetracycline will have an association constant substantially similar to or greater than that of tetracycline for tetR. Suitable analogs of tetracycline include doxycycline. Doxycycline exhibits similar dose response and induction characteristics with constructs of the invention, but has a longer half-life than tetracycline (48 hours vs. 24 hours respectively).

The coding sequence used in both ecdysone- and tetracycline-responsive constructs of the invention can be any sequence which encodes a NOS or a functional variant thereof. The phrase "nitric oxide synthase" is intended to include all naturally occurring forms of iNOS, nNOS and eNOS as well as variants which retain NOS activity, for example variants produced by mutagenesis techniques. Preferably the coding sequence encodes a NOS of mammalian origin for example rodent (including rat and mouse) or human. Most preferably the coding sequence encode the human iNOS (GenBank accession number: X73029, Coding sequence 226-3687), human nNOS (GenBank accession number: U17327, Coding sequence 686-4990) or human eNOS (GenBank accession number: M95296, Coding sequence 21-3632) or a functional variant of any one of those enzymes.

A functional variant of a NOS is any polypeptide which demonstrates NOS

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activity, for example a fragment of a NOS. A coding sequence which codes for a functional variant of a NOS may be, for example a fragment of a full length NOS coding sequence. A fragment may be of any length, so long as the polypeptide for which it codes has NOS activity.

5 A functional variant of a NOS typically comprises a sequence substantially similar to that of the naturally occurring form of the relevant NOS sequence. Thus, a functional variant of a NOS will generally have at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 98% or at least 99% sequence identity to the relevant NOS sequence, calculated over the full length of those sequences.

10 Thus, the coding sequence may be modified by nucleotide substitutions or deletions. For example up to 1, 2 or 3 to 10, 25, 50, 75 or 100 substitutions or deletions or combinations thereof may be made to produce a functional variant of a NOS. A polynucleotide encoding a NOS may alternatively or additionally be modified by one or more insertions and/or deletions and/or by an extension at either
15 or both ends. The modified polynucleotide generally encodes for a polypeptide which has NOS activity. Degenerate substitutions may be made and/or substitutions may be made which would result in a conservative amino acid substitution when the modified sequence is translated, for example as shown in the Table below. Amino acids in the same block in the second column and preferably in the same line in the
20 third column may be substituted for each other.

ALIPHATIC	Non-polar	G A P
		I L V
	Polar-uncharged	C S T M
		N Q
	Polar-charged	D E
		K R
AROMATIC		H F W Y

25 Sequence identity may be calculated as follows. The UWGCG Package provides the BESTFIT program which can be used to calculate identity (for example

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used on its default settings) (Devereux *et al* (1984) *Nucleic Acids Research* 12, p387-395). The PILEUP and BLAST algorithms can be used to calculate identity or line up sequences (typically on their default settings), for example as described in Altschul S. F. (1993) *J Mol Evol* 36:290-300; Altschul, S, F *et al* (1990) *J Mol Biol* 215:403-10. Software for performing BLAST analyses is publicly available through the National Centre for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>).

(ii) *Vectors*

Both types of construct of the invention can be incorporated into a recombinant replicable vector. The vector may be used to replicate the construct in a compatible host cell. A vector may also provide for expression of the NOS coding sequence when the vector is harboured by an appropriate host cell. The vectors may be for example, plasmid, virus or phage vectors provided with an origin of replication and optionally a regulator of the ecdysone-responsive promoter or promoter used in a tetracycline-responsive construct.

The vectors may contain one or more selectable marker genes, for example an ampicillin resistance gene for selection in bacterial cells or a G418 or a zeocin resistance gene for selection in mammalian cells.

(iii) *Cells*

Vectors of the invention, which incorporate an ecdysone-inducible construct, may be introduced into a suitable host cell by any appropriate transformation or transfection technique.

Preferably, the host cell will permit the expression of the NOS coding sequence. Thus, the cells may be chosen to be compatible with the said vector and may be for example bacterial, yeast, insect or mammalian cells. For NOS gene expression to be induced in the presence of ecdysone or an analog thereof, a cell harbouring an ecdysone inducible construct must preferably also be capable of expressing a functional ecdysone receptor.

As described above, the wild type *Drosophila* functional ecdysone receptor is a heterodimer of the ecdysone receptor (EcR) and the product of the ultraspiracle gene (USP). Thus cells of the invention may be capable of expressing EcR and USP.

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However, replacement of EcR's natural heterodimeric partner USP with its mammalian homologue retinoid X receptor gives a heterodimer which can give more potent induction of an ecdysone responsive promoter. Thus cells of the invention may be capable of expressing EcR and RXR. It will be clear that cells of the invention may be capable of expressing functional variants of either subunit of the heterodimer. Functional variants of EcR and USP/RXR are polypeptides which can heterodimerise with their partner and can, when heterodimerised, allow ecdysone-responsive dimerisation to occur. In some cases functional variants may bind to non-wild type EcREs. Examples of functional variants and modified EcREs are described in No *et al. Proc. Natl. Acad. Sci. USA*, 93: 3346-3351.

Preferred cells for use in the invention are human cells. Particularly preferred cells are EcR293 cells (Invitrogen, San Diego, CA; Catalogue No: R650-07; EcR293 is a derivative of the human fetal kidney cell line HEK293 (ECACC accession number 85/20602)). EcR293 cells are particularly suitable as they stably transformed with the vector pVgRXR. That vector is capable of expressing a functional variant of EcR, VgEcR, and RXR in mammalian cells and thus allows the expression of a functional ecdysone receptor. Other suitable cell lines include EcR-CHO and EcR-3T3 (Invitrogen, San Diego, CA; Cat. Nos: R660-07 and R680-07 respectively). Those two cell lines are stably transformed with the same vector, pVgRXR, as the EcR293 cell line.

Vectors of the invention, which incorporate an tetracycline-inducible construct, may be introduced into a suitable host cell by any appropriate transformation or transfection technique.

Preferably, the host cell will permit the expression of the NOS coding sequence in the presence of tetracycline or an analog thereof. Thus, the cells may be chosen to be compatible with the said vector and may be for example bacterial, yeast, insect or mammalian cells. For NOS gene expression to be regulated such that expression does not occur in the absence of tetracycline, a cell harbouring a tetracycline-inducible construct must preferably also be capable of expressing the tetracycline repressor protein (tetR) or a functional variant thereof.

A functional variant of tetR is a polypeptide which is similar to tetR and

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which remains capable of binding, as a homodimer, the TetO₂ site or a functional variant thereof and tetracycline or an analog thereof. Typically, the binding affinity of a functional variant sequence of tetR for the TetO₂ site or a functional variant thereof or tetracycline or an analog thereof may be substantially the same as the binding affinity of the tetR polypeptide for the TetO₂ site or a functional variant thereof or tetracycline or an analog thereof. Alternatively, a functional variant sequence may have a binding affinity which may be greater or less than that of the tetR polypeptide.

The *TetR* gene encodes a repressor protein of 207 amino acids with a calculated molecular weight of 23 kDa (Hillen and Berens, 1994, *supra*). A functional variant of tetR typically comprises an amino acid sequence substantially similar to that of the tetR sequence. Thus, a functional variant of a tetR will generally have at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 98% or at least 99% sequence identity to tetR, calculated over the full length of those sequences. The calculation of sequence identities is described above.

A functional variant of the tetR sequence may be a modified version of that sequence obtained by, for example, amino acid substitution or deletion. Up to 1, up to 10, up to 20, up to 50, up to 75, up to 100 or more amino acid substitutions or deletions or combinations thereof may be made to the tetR sequence to produce a functional variant of that sequence. Substitutions are preferably made which result in a conservative amino acid substitution, for example as shown in the Table above.

Preferred cells for use in the invention are human cells. Particularly preferred cells are T-REx cells (Invitrogen, San Diego, CA; Catalogue Nos: R710-07, R712-07, R714-07 and R716-07). T-Rex cells are particularly suitable as they stably transformed with the plasmid pcDNA6/TR which generates high level expression of the tetR polypeptide. However, any cell line can be used which expresses tetR or a functional variant thereof.

Methods for identifying a polynucleotide the expression of which is stimulated or inhibited by nitric oxide (NO)

The invention provides a method for identifying a polynucleotide, the

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expression of which is stimulated or inhibited by nitric oxide. Such a polynucleotide is a polynucleotide or polypeptide which is present in a greater or lesser amount in the presence of nitric oxide as when compared with the amount present in the absence of nitric oxide. Such polynucleotides may be referred to as differentially expressed polynucleotides.

A polynucleotide whose expression is stimulated or inhibited in the presence of nitric oxide will be so typically because of stimulation or inhibition of transcription and/or translation. The identification of a polynucleotide, the expression of which is stimulated or inhibited by nitric oxide will typically allow the isolation of the gene which corresponds to the polynucleotide identified. The term "gene" means a coding sequence together with its regulatory sequences such as promoters, enhancers, introns and terminators.

Typically two cell populations are provided and preferably both cell populations are cells which harbour a polynucleotide construct of the invention. One cell population will not have been exposed to NO and the other cell population will have been exposed to NO. The method could be carried out on the same cells ie. cells are cultured in the absence of ecdysone or tetracycline, an aliquot withdrawn and ecdysone or tetracycline added to the remaining cells. Alternatively, the method could be carried out on parallel populations of cells, cultured either in the absence or presence of ecdysone or tetracycline. Both arrangements may be carried out simultaneously.

RNA may then be isolated from the cells using any method known to those skilled in the art. Populations of mRNAs may be separated from total cellular RNA, the bulk of which may typically be rRNA and tRNA, using for example, an oligo(dT)-cellulose column. When a preparation of total cellular RNA is passed through such a column, mRNA molecules bind to the oligo(dT) by their poly(A) tails while the rest of the RNA flows on through the column. The bound mRNAs can then be eluted from the column using for example 10mM Tris and 1mM EDTA.

Optionally the mRNA may be converted into cDNA. Again methods for reverse transcription are well known to those in the art. Oligonucleotides, comprising stretches of, for example, 8 to 10 deoxythymidines may be used as

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primers for reverse transcriptase. Alternatively, random primed cDNA synthesis may be carried out. In that technique short oligonucleotide fragment of, for example, 6 to 10 nucleotides in length made up of many possible sequences are used as primers for the cDNA synthesis reaction. This technique may be suitable for isolating the 5' end of long messages. The product of both dT primed and random primed cDNA synthesis is an RNA-DNA hybrid. From that point several procedures may be used to convert the RNA-DNA hybrid to double stranded cDNA molecules suitable for cloning into appropriate vectors. For example, RNaseH nicking of the RNA strand followed by second strand cDNA synthesis.

mRNA or cDNA populations may be compared according to any method known to those skilled in the art. Generally, the comparison will be between two cDNA populations. For example, hybridisation-based, PCR-based or sequence-based techniques may be used. Hybridisation-based techniques that may be used include differential plaque-filter hybridization, subtraction cloning, cDNA array analysis and DNA microarray analysis. Suitable PCR-based techniques include differential display and representational difference analysis (RDA). Sequence-based techniques that may be used include serial analysis of gene expression (SAGE), expressed sequence tag (EST) analysis, massively parallel signature sequencing (MPSS), DNA sequencing chip analysis and mass spectrometry. See for example Kozian and Kirschbaum (1999) TIBTECH 17, 73-78.

(i) Hybridisation-based techniques

Differential plaque-filter hybridization allows the identification of specific differences in cloned cDNAs. The technique looks for differences in hybridisation when different cDNA populations are hybridised to replicates of a cDNA library. The technique has the limitation to the study of expression patterns of known genes.

Subtractive cDNA libraries may be generated by hybridizing an mRNA population of one origin to an mRNA of a different origin. Transcripts that do not find a complementary strand in the hybridisation step are then used for the construction of a cDNA library. That cDNA library allows the genes that are differentially expressed between the two populations of mRNA to be ascertained. A

number of refinements to this technique are possible, for example, the selective amplification of differentially expressed mRNAs via biotin- and restriction-mediated enrichment (SABRE). cDNAs derived from a tester population are hybridized against cDNAs from a driver (control) population. After a purification step specific for tester-cDNA-containing hybrids, tester-tester homohybrids are specifically amplified using an added linker, thus allowing the isolation of differentially expressed genes.

The above described hybridisation-based techniques all have the limitation that the results are unidirectional. That is, the result of such an experiment is the isolation of a population of differentially expressed genes. However, it is not possible to determine which population shows upregulation or downregulation of a particular differentially expressed gene. Further experimentation is required to determine the origin of the differential expression.

Labelled cDNAs, for example radioactively labelled or a non-radioactively labelled, for example with an antibody label or an enzyme label, may be hybridised to arrays of cDNAs. Such arrays comprise cDNAs spotted onto a solid matrix, for example modified cellulose or nylon so that each point of the array represents a known cDNA sequence. Comparison of identical arrays hybridized with two different populations of cDNAs therefore reveals genes which differentially expressed between the two cDNA populations. Typical cDNA arrays are available commercially, for example Clontech produce an array which has an expression profile of 588 known genes and GenomeSystems produce an array of 18,942 unrelated cDNA species covering about 20% of the expressed genes in the human genome. The latter array contains cDNA which correspond to genes with both known and unknown functions.

The cDNA array technique has been further developed with the introduction of DNA microarrays. Current DNA microarrays are systematically gridded at high density. Such arrays may be generated by using cDNAs (eg ESTs), PCR products or cloned DNA, which are linked to the surface of, for example, nylon filters, glass slides or silicon chips. DNA arrays may also be assembled from synthetic oligonucleotides, either by directly applying the synthesized oligonucleotides to the matrix or by photolithography. To determine differentially expressed genes, labelled

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cDNAs are hybridized to the DNA- or oligomer- carrying arrays. Indeed if two different fluorophores are used for labelling two different populations of cDNA to be tested the two populations can be hybridized simultaneously to the same array and compared at different wavelengths. The expression of 10, 000 genes or more can be analyzed on a single chip.

(ii) PCR-based techniques

PCR-based techniques have the advantage that differential gene expression may be analysed in a bidirectional manner and many cell populations can be analysed in parallel.

Differential display involves the amplification of cDNAs using a panel of random oligonucleotides. A typical protocol is as follows. DNase-treated total RNA of high purity is reverse transcribed using a $T_{11}XY$ primer ($X=A, C$ or G ; $Y=A, C, G$ or T) which serves as the template for subsequent PCR. The PCR is performed using a radiolabelled nucleotide, the same $T_{11}XY$ primer used for the reverse transcription and a set of random decamer primers. Each of these primer sets will amplify a subset of all cDNAs, resulting in the generation of up to a hundred cDNA fragments in one reaction tube. A portion of the PCR sample is then size fractionated by denaturing gel electrophoresis and the pattern of the amplified cDNAs is visualised autoradiographically. Comparisons of the cDNA band pattern lead to the identification of differentially amplified cDNAs, which can then be eluted from the gel, reamplified, cloned and sequenced.

Representational difference analysis takes advantage of both subtractive hybridization and PCR to analyze differential gene expression. In the first step, mRNA derived from two different populations, the tester and the driver (control) is reverse transcribed; the tester population is that in which the differential expression is expected to occur. Following digestion with a frequent-cutting restriction endonuclease, linkers are ligated to both ends of the cDNA. A PCR step then generates the initial representation of the different gene pools. The linkers of the tester and driver cDNA are digested and a new linker is ligated to the ends of the tester cDNA. The tester and driver cDNAs are then mixed in a 1:100 ratio with an

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excess of driver cDNA in order to promote hybridization between single-stranded cDNAs common in both tester and driver cDNA pools. Following hybridization of the cDNAs, PCR exponentially amplifies only those homoduplexes generated by the tester cDNA, via the priming sites on both ends of the double-stranded cDNA.

5

(iii) Sequence-based techniques

A number of sequence based techniques may be used to identify differentially expressed genes. However, in general these techniques rely on the use of databases of known gene sequences.

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In theory the use expressed sequence tags (ESTs) could be used to identify differentially expressed genes. That is all the mRNA encoding sequences can be identified in a particular tissue or cell type by sequencing all cDNA fragments isolated from that tissue or cell type at random. Comparison of the two tissue or cell types should reveal differentially expressed coding sequences. However, in practice this method is likely to be of limited use as a high through-put system. Sequencing all the ESTs from a particular tissue or cell type is likely to be a massive undertaking in even the simplest organisms.

15

Serial analysis of gene expression (SAGE) is a sequence-based approach to the identification of differentially expressed genes through comparative analyses. It allows the simultaneous analysis of sequences that derive from different cell populations or tissues. Three steps form the molecular basis for SAGE: (1) generation of a sequence tag (from 10 to 14 bp) to identify expressed transcripts; (2) ligation of sequence tags to obtain concatemers that can be cloned and sequenced; and (3) comparison of the sequence data to determine differences in expression of genes that have been identified by the tags.

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Methods for isolating stimulators or inhibitors of differentially expressed polynucleotides or the polypeptides which they encode

The invention provides methods for isolating stimulators or inhibitors of the differentially expressed polynucleotides identified by the methods described above or the polypeptides which the identified polynucleotides encode. Inhibitors or

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stimulators of differentially expressed polynucleotides are substances that inhibit or stimulate the expression the transcription of the polynucleotide into an mRNA or inhibit the translation of an mRNA into a protein. Inhibitors or stimulators of the polypeptide which differentially expressed polynucleotides encode are substances
5 that can inhibit or simulate the activity of such a polypeptide.

Clearly a particular substance may stimulate and/or inhibit transcription and/or translation and/or activity. Ultimately, however, the cumulative overall effect will be important, as in the majority of cases a polypeptide will be the active species. Generally if a substance inhibits transcription or translation the effective "activity" of
10 the corresponding gene will be inhibited. Although the activity per mole of polypeptide will be unaltered; the amount of polypeptide will be simply diminished.

Stimulators and inhibitors may be isolated using any suitable method. Typically, however, it will be convenient to use cells of the invention. Cells may be contacted with a test substance and ecdysone or an analog thereof or tetracycline or
15 an analog thereof under conditions in which in the absence of the test substance the expression of the polynucleotide or activity of the polypeptide is inhibited or stimulated in the presence of NO. The cells may then be assayed for the effect that the test substance has on expression of the differentially expressed polynucleotide or activity of the polypeptide. That is, the effect of the test substance on transcription,
20 translation and polypeptide activity may be assayed.

Suitable control experiments may also be carried out. For example, other genes may be assayed for in order to determine whether the test substance is a specific or general inhibitor or stimulator of transcription and/or translation and/or polypeptide activity.

25

Test substances

Suitable candidate substances for stimulators or inhibitors of differentially expressed polynucleotides or polypeptides include combinatorial libraries, defined chemical identities, peptide and peptide mimetics, oligonucleotides and natural
30 product libraries. The candidate substances may be used in an initial screen of, for example, ten substances per reaction, and the substance of these batches which show

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inhibition tested individually. Furthermore, antibody products (for example, monoclonal and polyclonal antibodies, single chain antibodies, chimaeric antibodies and CDR-grafted antibodies) which are specific for differentially expressed polypeptides may be used.

5 A stimulator or inhibitor of a differentially expressed polynucleotide or polypeptide is one which produces a measurable increase or reduction respectively in transcription and/or translation of the differentially expressed polynucleotide or activity of the polypeptide encoded by the polynucleotide in the assays described above.

10 Preferred inhibitors are those which inhibit expression of a polynucleotide and/or activity of a polypeptide by at least 10%, at least 20%, at least 30%, at least 40% at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95% or at least 99% at a concentration of the inhibitor of $1\mu\text{g ml}^{-1}$, $10\mu\text{g ml}^{-1}$, $100\mu\text{g ml}^{-1}$, $500\mu\text{g ml}^{-1}$, 1mg ml^{-1} , 10mg ml^{-1} , 100mg ml^{-1} .

15 Preferred stimulators are those which stimulate expression of a polynucleotide and/or activity of a polypeptide by at least 10%, at least 25%, at least 50%, at least 100%, at least, 200%, at least 500% or at least 1000% at a concentration of the activator $1\mu\text{g ml}^{-1}$, $10\mu\text{g ml}^{-1}$, $100\mu\text{g ml}^{-1}$, $500\mu\text{g ml}^{-1}$, 1mg ml^{-1} , 10mg ml^{-1} , 100mg ml^{-1} .

20 The percentage inhibition or stimulation represents the percentage increase or decrease in expression/activity in a comparison of assays in the presence and absence of the test substance. Any combination of the above mentioned degrees of percentage inhibition and concentration of stimulator or inhibitor may be used to define a stimulator or inhibitor of the invention, with greater stimulation or inhibition
25 at lower concentrations being preferred.

Therapeutic Use

Inhibitors or stimulators of transcription and/or translation of a polynucleotide identified by a method of the invention and/or of activity of a
30 polypeptide encoded by that polynucleotide may be useful in prophylaxis or therapy.

One of the genes isolated in screens carried out to identify polynucleotides

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which are differentially expressed in response to NO is the gene encoding DNA-PKcs. DNA-PK plays an important role in DNA repair and/or DNA damage signalling and DNA-PKcs is a member of the PI 3-kinase family, ie. it is a PI 3-kinase like kinase. Other polypeptides containing a PI 3-kinase-like domain are also implicated in DNA repair.

To test the biological significance of the increase in DNA-PKcs, we exposed cells to high doses of DNA damaging agents, such as NO donors, bleomycin, adriamycin, cisplatin, UV-C irradiation and X-ray irradiation.

NO generating cells, with increased levels of DNA-PK are fully protected against exposure to UV-C, bleomycin, adriamycin, cisplatin, X-ray irradiation and also to high concentrations of NO donors. A NOS inhibitor and a DNA-PK inhibitor can abolish this protection.

Therefore the invention also provides products containing an NOS inhibitor and a DNA damaging agent as a combined preparation for simultaneous, separate or sequential use in the treatment of cancer. The invention also provides products containing an inhibitor of a DNA repair enzyme and a DNA damaging agent as a combined preparation for simultaneous, separate or sequential use in the treatment of cancer.

Generally, preferred DNA repair enzymes are those which are PI 3-kinase like kinases. Also preferred are DNA repair enzymes that are upregulated in the presence of NO. DNA-PK is particularly preferred.

The condition of a patient suffering from a cancer can be improved by administration of products of the invention. A therapeutically effective amount of products of the invention may be given to a patient in need thereof.

The invention also provides use of an NOS inhibitor in the manufacture of a medicament for use with a DNA damaging agent in the treatment of cancer. The invention additionally provides use of a DNA repair enzyme inhibitor, for example a DNA-PK inhibitor, in the manufacture of a medicament for use with a DNA damaging agent in the treatment of cancer.

DNA damaging agents suitable for use in the invention include substances which are DNA alkylating and/or cross-linking agents, substances which are DNA

binding/cleaving agents and radiotherapeutic agents, for example X-ray irradiation.

Substances which are DNA alkylating and/or cross-linking agents include nitrosoureas, nitrogen mustards, mitomycins and platinum coordination compounds. Such substances typically have the ability to react covalently with DNA bases and to form inter- and intrastrand DNA cross-links. These compounds may also be responsible for the alkylation of proteins and protein-DNA linkages. The resulting lesions produced in the DNA result in the disruption of cell growth and function, ultimately leading to cell death.

Suitable nitrosoureas include carmustine USP (BiCNU), lomustine USP (CeeNU), tauromustine and streptozocin USP (Zanosar).

Suitable nitrogen mustards include cyclophosphamide USP (Cytoxan), ifosfamide (Ifex), mesna USP (Mesnex), mechlorethamine hydrochloride USP (Mustargen), chlorambucil USP (Leukeran), melphalan USP (Alkeran) and thiotepa USP (Thiotepa).

Suitable mitomycins include mitomycin C USP (Mutamycin), BMY-25067 and KW2149.

Suitable platinum coordination compounds include cisplatin USP (Platinol) and carboplatin USP (Paraplatin).

Substances which are DNA binding/cleaving agents (DNA interactive agents) may bind to DNA either as intercalators or as minor groove binders, hence inhibiting DNA dependent RNA synthesis. Such substances may also cleave DNA by forming free radicals in the immediate vicinity of the sugar-phosphate backbone. Activity as antitumor agents is typically related to the ability to induce irreparable lesions in DNA. For example, one suitable substance bleomycin generates oxygen free-radical species, whereas another suitable substance, esperamicin A₁, generates aryl diradical species, which abstract hydrogen atoms directly from the deoxyribose backbone.

Suitable DNA interactive substances include danorubicin hydrochloride USP (Cerubidine), doxorubicin USP (Adriamycin), idarubicin hydrochloride (Idamycin), mitoxanthrone hydrochloride USP (Novantrone), bleomycin sulfate USP (Blenoxane), esperamicin A₁, Adozelesin (U73, 975), dactinomycin USP (Cosmegen), plicamycin USP (Mithracin), and procarbazine hydrochloride USP

(Matulane).

It will be apparent to those skilled in the art that analogs of the above mentioned DNA damaging agents may also be suitable for use in the invention.

5 An NOS inhibitor is a substance which inhibits transcription and/or translation of an NOS gene and/or inhibits activity of a NOS enzyme. Any pharmaceutically acceptable inhibitor of NOS can be used in the present invention.

Typically, an inhibitor of a NOS enzyme is used. Competitive, non-competitive, reversible and irreversible inhibitors are suitable. The inhibitor may inhibit iNOS, eNOS and/or nNOS. Preferably, the inhibitor will selectively inhibit
10 the NOS isoform expressed in the tumor to be treated.

Suitable inhibitors include L-arginine analogues, thiocitrullines, indazole derivatives, imidazole derivatives, hydrazine derivatives, thioureas, thiazoles, biotin derivatives and phenyl-substituted thiopene amidines.

Examples of suitable L-arginine analogues include methyl-L-arginine, N^G-nitro-L-arginine methyl ester (L-NAME), N^G-monomethyl-L-arginine (L-NMMA),
15 N^G-amino-L-arginine (L-NAA), N^w,N^w-dimethyl-L-arginine (ADMA), N^w,N^{w2}-dimethyl-L-arginine (SDMA), N^w-ethyl-L-arginine (L-NEA), N^w-methyl-L-homoarginine (L-NMHA), N^w-nitro-L-arginine (L-NOARG), N^δ-iminoethyl-L-ornithine (L-NIO), N^δ-iminoethyl-L-lysine (L-homo-NIO) and L-canavanine (L-
20 CAN).

Examples of suitable thiocitrullines include S-methyl-L-thiocitrulline (SMTTC), L-thiocitrulline (L-TC) and L-S-ethyl-thiocitrulline (Et-TC).

Examples of suitable indazole derivatives include indazole and 7-substituted indazoles such as 7-nitroindazole and 3-bromo-7-nitroindazole.

25 Examples of suitable hydrazine derivatives include aminoguanidine.

Examples of suitable imidazole derivatives include phenyl substituted imidazoles such as 1-phenyl-imidazole.

Examples of suitable thioureas include S-methylisothiurea sulphate, δ-(S-methylisothioureido)-L-norvaline (L-MIN), S-ethylisothiurea (SETU) and S-isopropylisothiurea (SIPT).
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Examples of suitable thiazoles include 2-amino-thiazole and 2-amino-4,5-

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dimethyl thiazole.

Examples of suitable biotin derivatives include 2-iminobiotin.

The above NOS inhibitors are commercially available, or may be made by analogy with known methods.

5 The inhibitor may be a pharmaceutically acceptable salt of one the above compounds. Suitable salts include salts with pharmaceutically acceptable acids, both inorganic acids such as hydrochloric, sulphuric, phosphoric, diphosphoric, hydrobromic or nitric acid and organic acids such as citric, fumaric, maleic, malic, ascorbic, succinic, tartaric, benzoic, acetic, methanesulphonic, ethanesulphonic,
10 benzenesulphonic or p-toluenesulphonic acid. Salts may also be formed with pharmaceutically acceptable bases such as alkali metal (eg sodium or potassium) and alkali earth metal (eg calcium or magnesium) hydroxides and organic bases such as alkyl amines, aralkyl amines or heterocyclic amines.

Inhibitors of NOS can be identified by:

15 (a) contacting a candidate compound with NOS and a substrate and co-factor therefor, under conditions under which NOS activity, in the absence of an inhibitor, would be expected to occur, and

 (b) determining whether, or to what extent, NOS activity takes place.

 A suitable such assay for identifying inhibitors of NOS is a microtiter plate
20 assay in which NOS activity is measured by determining the change in absorbance as NADPH is converted to NADP⁺. This assay comprises:

 (a) adding a candidate compound, a known NOS inhibitor (for example L-NMMA) and a buffer solution to separate microtiter wells;

 (b) adding to each well NOS enzyme, cofactors therefor, L-arginine and
25 buffer, and

 (c) determining the change in absorbance in each well.

 Typically, the buffer is a HEPES buffer capable of maintaining a pH of about 7, preferably about 7.4. The cofactors comprise oxyhemoglobin, NADPH and BH₄. They may also comprise CaCl₂, MgCl₂, FMN, FAD and/or CaM.

30 The NOS may be a naturally occurring form of eNOS, iNOS, or nNOS or may be a variant which retains NOS activity, for example variants produced by

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mutagenesis techniques. NOS used in the assay is preferably of mammalian origin, for example rodent (including rat and mouse) or primate (such as human).

Preferably, the NOS is of human origin.

The NOS may be obtained from mammal cellular extracts or produced recombinantly from, for example, bacteria, yeast or higher eukaryotic cells including mammalian cell lines and insect cell lines. Preferably, NOS used in the assay is recombinant. More preferably, it is obtained by expression in Sf21 cells according to the methodology in Charles *et al.*, *Methods in Molecular Biology* (edited by M.A. Titheradge, Humana Press, Totowa), vol 100, pgs 51-60.

Step (c) of the assay may be carried out by reading the difference in absorbance between 420 and 405 nm. Typically, this is done by a spectrophotometer. Comparison of the well containing the candidate compound with the control wells containing a known NOS inhibitor (100% inhibition) and no inhibitor (0% inhibition) allows % inhibition achieved by the candidate compound to be calculated.

A microtiter assay as set out above is described in detail in Dawson & Knowles, *Methods in Molecular Biology* (edited by M.A. Titheradge, Humana Press, Totowa), vol 100, Chapt. 22, pgs 237-242.

Any compound which is identified as an NOS inhibitor using an assay as described above can be used in the present invention. The NOS inhibitors used in the present invention typically achieve at least 50% NOS inhibition, more preferably at least 80% NOS inhibition. Ideally, they achieve substantially complete NOS inhibition.

A DNA repair enzyme inhibitor is a substance which inhibits transcription and/or translation of a gene encoding a DNA repair enzyme and/or inhibits activity of a DNA repair enzyme itself. Any pharmaceutically acceptable inhibitor of DNA repair enzyme can be used in the present invention.

Typically, an inhibitor of a DNA repair enzyme itself is used. Competitive, non-competitive, reversible and irreversible inhibitors are suitable. The inhibitor may inhibit one subunit of a DNA repair enzyme, for example the catalytic subunit of DNA-PK. Suitable inhibitors include wortmannin, OK-1035, LY294002, quercitin,

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quercitrin and rutin and analogs and derivatives thereof. These inhibitors are all inhibitors of PI 3-kinase like kinases and are commercially available, or may be made by analogy with known methods.

The inhibitor may be a pharmaceutically acceptable salt of one the above compounds. Suitable salts include salts with pharmaceutically acceptable acids, both inorganic acids such as hydrochloric, sulphuric, phosphoric, diphosphoric, hydrobromic or nitric acid and organic acids such as citric, fumaric, maleic, malic, ascorbic, succinic, tartaric, benzoic, acetic, methanesulphonic, ethanesulphonic, benzenesulphonic or p-toluenesulphonic acid. Salts may also be formed with pharmaceutically acceptable bases such as alkali metal (eg sodium or potassium) and alkali earth metal (eg calcium or magnesium) hydroxides and organic bases such as alkyl amines, aralkyl amines or heterocyclic amines.

Inhibitors of the DNA repair enzyme DNA-PK are particularly suitable for use in the present invention and can be identified by, for example:

(a) Growing EcR293 clone 11 cells for 24h in the presence of muristerone A. An equal number of untreated cells are also prepared. Cells treated with muristerone A are checked for NO generation by use of the Griess reaction;

(b) Cells are trypsinised and seeded into 96 well plates. Three sets of plates are produced for each experiment: 2 sets of muristerone A treated cells; and one plate of untreated cells;

(c) The two sets of cells that have been given muristerone A are treated with UV-C as described in the Example below and the control cells are left untreated;

(d) A library of test compounds is added to one set of 96-well plates that have been treated with muristerone A and exposed to UV-C. The same test compounds are added to the untreated control cells. The other 96-well plate that has been treated with muristerone A and exposed to UV-C receives no test compounds and is used as a control;

(e) Cells exposed to UV-C that have not received muristerone A will undergo 95% cell killing. Those cells receiving muristerone A treatment will generate NO, up-regulate DNA-PK, and be protected against the damaging effects of UV-C (95% protected). Test compounds that inhibit DNA-PK will result in

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increased cell death;

(f) A simple colourimetric assay for LDH (lactate dehydrogenase) can be carried out as described in the Example below to determine cell viability. Any test compound that inhibits DNA-PK will result in a reduction of cell survival (ie. an increase in the LDH assay); and

(g) Candidate compounds isolated in step (f) can be assayed against the DNA-PK enzyme to determine whether they are DNA-PK inhibitors or PI 3-kinase like kinase inhibitors.

The above assay may alternatively be carried out using a cell line which expresses NOS under the control of an tetracycline-responsive promoter.

Any compound which is identified as a DNA-PK inhibitor using an assay as described above can be used in the present invention.

The DNA repair enzyme inhibitors used in the present invention typically achieve at least 50% inhibition of a DNA repair enzyme, more preferably at least 80% inhibition of a DNA repair enzyme. Ideally, they achieve substantially complete inhibition of a DNA repair enzyme.

Products of the invention may be used in the treatment of any cancer. The particular cancer to be treated will typically depend on the particular DNA damaging agent to be used. For example, typically, cisplatin may be used to treat metastatic testicular tumours, metastatic ovarian tumours and advanced bladder cancer. The products of the invention may also be used in the treatment of breast cancer, ovarian cancer, hepatoma or melanoma.

Use of radiotherapy, for example X-ray irradiation, with a NOS inhibitor or a DNA repair enzyme inhibitor according to the invention, may be used in the treatment of any suitable cancer, including breast, lung, cervical, colorectal, head and neck cancers.

The invention also provides a method of treatment of a host suffering from retrovirus infection, for example HIV infection, comprising administration of a therapeutically effective amount of an NOS inhibitor. Optionally, an effective amount of a PI-3 kinase like kinase, for example DNA-PKcs, may be co-administered.

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Therefore, the invention also provides products containing an NOS inhibitor and a PI-3 kinase like kinase as a combined preparation for simultaneous, separate or sequential use in the treatment of retroviral infection. The condition of a patient suffering from retroviral infection can be improved by administration of products of the invention. A therapeutically effective amount of products of the invention may be given to a patient in need thereof.

The invention also provides use of an NOS inhibitor in the manufacture of a medicament for use in the treatment of retroviral infection. The invention additionally provides use of an NOS inhibitor in the manufacture of a medicament for use with a PI-3 kinase like kinase in the treatment of retroviral infection.

A host suffering from retroviral infection may also be treated, for example separately, sequentially or simultaneously, with any other anti-retrovirus agent when carrying out the above method or use or when using the above products.

DNA damaging agents and NOS or DNA repair enzyme inhibitors may be administered in a variety of dosage forms. Thus, they can be administered orally, for example as tablets, troches, lozenges, aqueous or oily suspensions, dispersible powders or granules. The DNA alkylating and/or cross-linking agents and NOS or DNA repair enzyme inhibitors may also be administered parenterally, either subcutaneously, intravenously, intramuscularly, intrasternally, transdermally or by infusion techniques. The DNA alkylating and/or cross-linking agents and NOS or DNA repair enzyme inhibitors may also be administered as suppositories. A physician will be able to determine the required route of administration for each particular patient.

The formulation of a DNA alkylating and/or cross-linking agent and an NOS or a DNA repair enzyme inhibitor for use in preventing or treating cancer will depend upon factors such as the nature of the exact DNA alkylating and/or cross-linking agent and NOS or DNA repair enzyme inhibitor, whether a pharmaceutical or veterinary use is intended, etc. An NOS or a DNA repair enzyme inhibitor and a platinum coordination compound may be formulated for simultaneous, separate or sequential use.

A DNA alkylating and/or cross-linking agent and an NOS or a DNA repair

enzyme inhibitor is typically formulated for administration in the present invention with a pharmaceutically acceptable carrier or diluent. The pharmaceutical carrier or diluent may be, for example, an isotonic solution. For example, solid oral forms may contain, together with the active compound, diluents, e.g. lactose, dextrose, 5 saccharose, cellulose, corn starch or potato starch; lubricants, e.g. silica, talc, stearic acid, magnesium or calcium stearate, and/or polyethylene glycols; binding agents; e.g. starches, arabic gums, gelatin, methylcellulose, carboxymethylcellulose or polyvinyl pyrrolidone; disaggregating agents, e.g. starch, alginic acid, alginates or sodium starch glycolate; effervescing mixtures; dyestuffs; sweeteners; wetting 10 agents, such as lecithin, polysorbates, laurylsulphates; and, in general, non-toxic and pharmacologically inactive substances used in pharmaceutical formulations. Such pharmaceutical preparations may be manufactured in known manner, for example, by means of mixing, granulating, tableting, sugar-coating, or film coating processes.

Liquid dispersions for oral administration may be syrups, emulsions and 15 suspensions. The syrups may contain as carriers, for example, saccharose or saccharose with glycerine and/or mannitol and/or sorbitol.

Suspensions and emulsions may contain as carrier, for example a natural gum, agar, sodium alginate, pectin, methylcellulose, carboxymethylcellulose, or polyvinyl alcohol. The suspensions or solutions for intramuscular injections may 20 contain, together with the active compound, a pharmaceutically acceptable carrier, e.g. sterile water, olive oil, ethyl oleate, glycols, e.g. propylene glycol, and if desired, a suitable amount of lidocaine hydrochloride.

Solutions for intravenous or infusions may contain as carrier, for example, sterile water or preferably they may be in the form of sterile, aqueous, isotonic saline 25 solutions.

A therapeutically effective amount of a DNA alkylating and/or cross-linking agent and of an NOS or DNA repair enzyme inhibitor is administered to a patient. The dose of a DNA alkylating and/or cross-linking agent and of an NOS or a DNA repair enzyme inhibitor may be determined according to various parameters, 30 especially according to the substance used; the age, weight and condition of the patient to be treated; the route of administration; and the required regimen. Again, a

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physician will be able to determine the required route of administration and dosage for any particular patient. A typical daily dose is from about 0.1 to 50 mg per kg of body weight, according to the activity of the specific inhibitor, the age, weight and conditions of the subject to be treated, the type and severity of the degeneration and the frequency and route of administration. Preferably, daily dosage levels are from 5 mg to 2 g.

Use of radiotherapy according to the invention may be carried out according to techniques well known in the art. A physician will be able to determine suitable administration and dosage regimes for each particular patient

The following Example illustrates the invention:

EXAMPLE

Materials and methods

Unless indicated otherwise, the methods used are standard biochemical techniques. Examples of suitable general methodology textbooks include Sambrook *et al.*, Molecular Cloning, a Laboratory Manual (1989) and Ausubel *et al.*, Current Protocols in Molecular Biology (1995), John Wiley & Sons, Inc.

Subculture Procedure

All of the operations were carried out under strict aseptic conditions in a laminar flow hood. The medium was removed from near-confluent flasks and gently washed twice with serum-free DMEM (Dulbecco's Modified Eagle's Medium). A trypsin/versene mix was added to the cells and they were incubated for 5-10 min. until the cells had detached. Once the cells had detached they were resuspended in pre-warmed (37°C) serum-free DMEM and then pelleted by centrifugation at 1000rpm for 5min. It was essential to wash the cells to remove the residue of the trypsin/versene (serum-free DMEM was used for washing). The supernatant was removed and the cells gently resuspended in 15ml of 90% DMEM with glutamine, 10% fetal bovine serum (FBS, cell culture grade) that had been pre-warmed to 37°C. The cells were then transferred to three or four T-25 flasks (or equivalent) and placed

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in a humidified, 37°C, 5% CO₂ incubator. It typically took 3-4 days for cell cultures to reach 80-90% confluency. Media were changed 2 times a week and cells subcultured at a ratio of 1:3 to 1:4 when they reached 80-90% confluency. Cells were subcultured when approaching confluency to avoid the accumulation of floating and dead cells. Cells were frozen for storage in 95% FBS and 5% DMSO.

Induction using Muristerone A

Cells could be maintained in non selection medium for 2-3 weeks without losing inducibility following muristerone A treatment. Non-selection medium was used by choice, although selection medium worked equally well. Cells were seeded at 1-2 x 10⁵/ml concentration for 12 well or 96 well plates. Semi-confluent or confluent plates or flasks were used for induction. Figure 1(a) shows overnight induction in 12 well plates (3-30 hours) following the addition of different doses of muristerone A. Nitrate concentrations are measured using the Griess Reaction (see below).

Western blot analysis

Cells were pelleted at 200g, followed by two washes in ice-cold PBS, pH 7.2, then resuspended in the extraction buffer (50mM NaF, 20mM Hepes (pH 7.8), 450mM NaCl, 25% (vol/vol) glycerol, 0.2mM EDTA, 0.5mM dithiothreitol, 0.5mM phenylmethylsulfonyl fluoride, leupeptin (0.5µg/ml), protease inhibitor (0.5µg/ml), trypsin inhibitor (1.0µg/ml), aprotinin (0.5µg/ml), bestatin (40µg/ml)) and left on ice for 10 min. Following centrifugation at 10,000g for 10 min at 4°C, the supernatant was collected and the cell extract assayed for protein using the BCA kit (Pierce). One fifth of a volume of five times sample buffer (0.25M Tris-HCL (pH6.8), 0.4M DTT, 5% SDS, 0.5% bromophenol blue, 50% glycerol) was added to each sample and boiled for 5 min prior to storage at -70°C. Electrophoresis was carried out on 6% SDS polyacrylamide gels with 25µg samples. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Amersham) and immunoblotting carried out with the appropriate antibody using ECL (Amersham). Where necessary, blots were stripped in 62.5mM Tris-HCL 100mM β-mercaptoethanol/ 2% SDS, (pH

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6.7) and reprobed with different antibodies.

Northern Blotting

Poly(A) + mRNA was isolated using a micro-fastrack mRNA purification kit (Invitrogen), separated by electrophoresis and transferred onto Hybond N membrane (Amersham). Phosphoimaging (BAS1000, Fujix) was used to quantify the signals using the MacBas image analysis software.

NOS activity assay

Griess Reaction (Green *et al.*, 1982, Analysis of nitrate, nitrite and [^{15}N] nitrate in biological fluids, *Anal. Chem.* 126, 131-138): NOS activity was determined for both intact cells and their lysates. For intact cells, 100 μl of the culture medium was mixed with 100 μl of Griess reagent (1:1 mixture of 1% sulfanilamide in 5% H_3PO_4 and 0.1% naphthylethylenediamine dihydrochloride in water) for 10 min at room temperature and the absorbance at 543 nm was recorded. A serial dilution of sodium nitrite was used as a standard.

For enzyme assays on cell lysates, 100 μg of lysate was mixed with 100 μl reaction reagent from the NOS detect system (Stratagene NOS detect kit, Cat. No. 204500). The kit measures the conversion of [^{14}C]arginine to [^{14}C] citrulline, and is specific for the NOS pathway.

Whole Cell and nuclear DNA-PK assay

DNA-PK 'pulldown' kinase assays were carried out (Finnie *et al.*, 1995, Proc. Natl. Acad. Sci. USA 93, 2442-2447) using pre-swollen double stranded DNA (dsDNA) cellulose (Sigma) in a total volume of 50ml of 'Z' buffer (25mM Hepes/KOH, pH 7.9, 50mM KCL, 10mM MgC12, 20% glycerol, 0.1 Nonidet P40, 1mM dithiothreitol). The dsDNA cellulose was then washed twice with 1ml of 'Z' buffer and resuspended in 50ml of 'Z' buffer. Samples were divided into two or three aliquots. 0.5ml of γ^{32} PATP (3000Ci/mmol) was added and kinase assays were carried out in the presence or absence of 4 nmol of peptide (0.2mM). Reactions were then stopped and analysis by spotting on to phosphocellulose paper, washing and

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counting as described. The sequences of the modified p53 N-terminal substrate (wt) and mutant p53 peptides are: EPPISQ~~EE~~AFALLKK and EPPLSEQ~~EE~~AFALLKK, respectively. All assays were performed multiple times with at least three different extract preparations. The reproducibility of the DNA-PK pulldown peptide assay is generally less than $\pm 10\%$. Nuclear extracts were prepared as above and DNA-PK activity analyses using the SignaTECT DNA Dependent Protein Kinase Assay System (Promega).

Cell Culture and UV-C irradiation

EcR293 clone 11 cells were cultured and treated with muristerone A as described above. Following treatment with muristerone A for 24h, cells were washed and exposed to UV-C (254nm) at a dose of 120mJ/cm² with a UV-Stratolinker 1800 (Stratagene). Fresh medium was added and the cells incubated for a further 24hr on 6-well flat-bottom microtiter plates.

X-ray irradiation

Exponentially growing untransfected cells and EcR293 clone 11 cells, either untreated or treated with 10 μ M muristerone A were X-ray irradiated using a Pantak X-ray machine at 320Kv and 10mA, at a dose rate of 6 Gy Min⁻¹. Cells (5,000-15,000 per well) were plated in 6-well Falcon plates and incubated for 2-3 weeks. After staining with methylene blue, colonies of >50 cells were counted under magnification. The "surviving fraction" was determined by direct comparison of colony numbers from untreated wells with those from X-ray-irradiated wells.

Cell Survival Assay

Trypan blue (0.4%) solution (Gibco BRL) was used to stain dead cells in which loss of viability is recognised by membrane damage resulting in penetration of the dye. The viability is expressed as per cent viable cells in the population. The cytotoxicity detection kit (Boehringer) is based on the measurement of lactate dehydrogenase (LDH) activity released into the culture supernatants was measured with a 30-min couple enzymatic assay which results in the conversion of a

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tetrazolium salt into a red formazan product that is read at 490nm in an automatic plate reader (Emax. Molecular Device, Sunnyvale, CA). Measurement of cytotoxicity by ATP was carried out with ATPlite-M (Packard) reagents. Nuclear fragmentation assays were determined with TdT (terminal deoxynucleotidyl transferase) mediated dUTP-biotin nick end labelling (TUNEL) using the tumorTACS kit (R&D systems). For each sample, a minimum of 500 cells were counted. Labelled nuclei are expressed as a percentage of the total number.

Results

10 **Generation of NOS transfected cell lines under the control of an ecdysone-responsive or a tetracycline-responsive promoter**

Three plasmids were generated, each of which expressed one of the human NOS isoforms under the control of an ecdysone-responsive promoter. An additional plasmid was constructed placing the iNOS cDNA under the control of a tetracycline-regulated promoter.

(I) pIND-hiNOS-f (Figure 1a)

4164bp of the human iNOS cDNA (GenBank accession number: X73029, Coding sequence 226-3687) was cut from its original vector (Bluescript KS) using the restriction endonucleases, *KpnI* and *SpeI* and cloned into pIND (Invitrogen, San Diego, CA; Catalogue No: V705-20) which had been cut with *KpnI* and *XbaI*. The resulting plasmid was sequenced to confirm that the cDNA had inserted in the correct orientation.

25 (II) pIND-hnNOS-f (Figure 1b)

5kb of the human nNOS cDNA (GenBank accession number: U17327, Coding sequence 686-4990) was cut from its original vector (Bluescript KS) using the restriction endonucleases, *XbaI* and *KpnI* and cloned into pIND (Invitrogen, San Diego, CA; Catalogue No: V705-20) which had been cut with *NheI* and *KpnI*. The resulting plasmid was sequenced to confirm that the cDNA had inserted in the correct orientation.

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(III) pIND-heNOS-f (Figure 1c)

The wild type human eNOS (GenBank accession number: M95296, Coding sequence 21-3632) was cut from its original vector (Bluescript KS) using the restriction endonucleases, *HindIII* and *NotI* and cloned into pIND (Invitrogen, San Diego, CA; Catalogue No: V705-20) which had been cut with *HindIII* and *NotI*. The resulting plasmid was sequenced to confirm that the cDNA had inserted in the correct orientation.

(IV) pTet-hiNOS-f (Figure 1d)

The human iNOS cDNA (GenBank accession number: X73029, Coding sequence 226-3687) was cut from its original vector (Bluescript KS) using the restriction endonucleases, *KpnI* and *SpeI* and cloned into pcDNA4/TO (Invitrogen, San Diego, CA) which had been cut with *KpnI* and *XbaI*. The resulting plasmid was sequenced to confirm that the cDNA had inserted in the correct orientation.

The pIND plasmid contains 5 modified EcREs called E/GREs which bind a modified functional ecdysone receptor. That modified functional ecdysone receptor can be expressed by another plasmid, pVgRXR (Invitrogen, San Diego, CA; Catalogue Number: V730-20). pVgRXR constitutively expresses a heterodimeric receptor comprising a modified ecdysone receptor (VgEcR) and RXR. Thus, a cell transformed with pVgRXR and one of the three plasmids described above (I, II or III) will express NOS in the presence of ecdysone or an analog thereof. In the presence of ecdysone the functional ecdysone receptor binds to the E/GREs and transcription of the NOS cDNA is initiated.

The plasmid pIND-hiNOS-f was used to transfect a human fetal kidney cell line, EcR293 (Invitrogen, San Diego, CA; Catalogue No: R650-07), which is stably transformed with pVgRXR. Transfections were carried out using Superfect reagent (Qiagen) and transfectants were isolated following double selection on G418 (400µg/ml) and zeocin (250µg/ml) for 14 days.

Thus, cells were isolated which constitutively expressed the subunits of a functional ecdysone receptor, RXR/VgEcR and the human iNOS cDNA under the

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control of an ecdysone-inducible promoter.

The T-Rex system (Invitrogen, San Diego, CA; Catalogue No: K1020-01) is a tetracycline-regulated mammalian expression system that uses regulatory elements from the *E. coli* Tn10-encoded tetracycline resistance operon. The pcDNA4/TO plasmid allows expression of a gene of interest under the control of the strong human cytomegalovirus immediate-early (CMV) promoter and two tetracycline operator 2 (TetO₂) sites. The pcDNA6/TR plasmid expresses high levels of the *TetR* gene under the control of the human CMV promoter. Thus, a cell transformed with pcDNA6/TR and the plasmid described above (IV) will express NOS in the presence of tetracycline or an analog thereof. When present, tetracycline binds to tetR which undergoes a conformational change such that it dissociates from the TetO₂ sites. Expression of the iNOS gene is then induced, driven by the CMV promoter.

The plasmid pTet-hiNOS-f was used to transfect the cell line T-REx-293 ((Invitrogen, San Diego, CA; Catalogue No: R710-07). The T-REx-293 cell line is a human embryonic kidney 293 cell line which has been transfected with the pcDNA6/TR plasmid and thus generates high level expression of the tetracycline repressor protein (TetR). Transfections were carried out using conditions as described above for the muristerone A inducible constructs. Transfectants were isolated following selection on zeocin (200µg/ml) for the human iNOS cDNA expressing plasmid and blasticidin (5µg/ml) for the tetR expressing plasmid.

Thus, cells were isolated which constitutively expressed the tetracycline repressor protein (tetR) and the human iNOS cDNA in the presence of tetracycline.

Isolation and characterization of ecdysone-responsive and tetracycline-responsive human cell lines

(A) Isolation of a panel of ecdysone-responsive human cell lines and determination of NOS activity

A panel of 20 G418/zeocin-resistant clones were examined for their ability to generate NO. A total of 5 were identified that could be induced to produce NO at varying levels after treatment with 100µM muristerone A for 24 hours. NOS activity

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was determined in both intact cells and in cell lysates. For intact cells, the Griess reaction was used to determine the concentration of NO in 100µl of culture medium. For enzyme assays, 100µg of cell lysate was mixed with 100µl reaction reagent from NOS detect system (Stratagene NOS detect kit, Cat. No.204500). The kit measures the conversion of [¹⁴C] arginine to [¹⁴C] citrulline, and is specific for NOS. For a typical experiment, transfectants were plated out on 12 well Falcon tissue culture plates at a cell density of 1×10^5 /ml and nitrite was measured by the Griess reaction. Muristerone A (Invitrogen) was added at a final concentration of 100µM to specific wells, and after induction for 24hr, 100µl of culture supernatant was used to measure nitrite concentration using the Griess reagent. The results are reported as the average of assays run on triplicate wells. Well-to-well variation was less than 10%.

(B) Time and dose response of EcR293 clone 11 cells generating NO

One of the transfectants, clone 11, was selected for further study. Cells were grown with varying concentrations of muristerone A, and at different time intervals, supernatants were taken and the Griess reaction was used to measure the nitrite concentration. The results are reported as the average of assays run in triplicate. Well-to-well variation was less than 10%. See Figure 2a.

(i) Muristerone A-dependent expression of the human iNOS gene. Northern blot analysis was carried out with 2µg of polyA⁺ RNA isolated from cells which had been treated with muristerone A for 24 hr. A human iNOS cDNA probe was used to detect the presence of a 4kb band in mRNA extracted from cells treated with either 1µM or 10µM muristerone A. Human β-actin mRNA was used as a loading control. See Figure 2b.

(ii) Western blot of iNOS protein expression was carried out on untreated control cells or cells treated with 10µM muristerone A. The cells were harvested and 20µg of whole cell extracts loaded on to a 6% polyacrylamide gel. Following electrophoresis, the proteins were transferred to a filter and probed with a polyclonal antibody raised against the 7 C-terminal residues of human iNOS: Cyc-Arg-Nle-Orn-(Ser-Leu-Glu-Met-Ser-Ala-Leu). Filters were stripped and an antibody against human alpha-tubulin. (Insight Biotchnology) was used as a control. See Figure 2b.

(C) Isolation of a panel of tetracycline-responsive human cell lines and determination of NOS activity

A panel of 4 tetracycline-regulated iNOS expressing cell lines were isolated,
5 designated clone 1, clone 2, clone 5 and clone 22.

In the absence of the inducer tetracycline the iNOS transfectants were unable to express mRNA as the two Tet operator sites (TetO₂) are occupied by the repressor protein effectively blocking transcription. When tetracycline is added to the culture medium, it binds to the TetR protein and changes its conformation. The altered
10 conformation of the repressor is unable to bind the operator sites, and consequently iNOS can be expressed. Nitrite concentrations were determined using the Griess reaction (see figure 3).

NO-induced up-regulation of DNA-PKcs

15 Previous work has demonstrated that treatment of cells with NO can lead to an increase in the accumulation of wild type p53 correlating with the transcriptional up-regulation of the p21^{WAF1} cyclin-dependent kinase inhibitor. NO can also transcriptionally down-regulate expression of vascular endothelial growth factor (VEGF). Our experiments confirmed the previously reported findings that an
20 increase in NO concentration results in a decrease in VEGF and an increase in both wild type p53 and p21^{WAF1}.

In order to examine the role of NO on gene expression we used a differential display strategy involving differential hybridisation of mRNA derived probes to normalized cDNA arrays. Two different populations of mRNA were isolated, one
25 from EcR293 clone 11 cells, and the other from the same cells expressing NO following treatment with 10 μ M muristerone A for 24 hr. cDNA probes prepared from the mRNA isolated from these cells were radioactively labelled, and used to hybridize to normalized human cDNA expression arrays (Atlas Human cDNA Expression Array I, Clontech), containing an expression profile of 588 genes.

30 Analysis of the resulting differential hybridization pattern reveals signals for a number of cDNA sequences with different intensities upon generation of NO. DNA-

PKCs was found to be one of the candidate genes for which expression is higher in cells expressing NO.

To confirm that the changes in hybridization signal on the filter array corresponded to changes in mRNA abundance, Northern blotting experiments were carried out. Figure 4a shows a 13kb band is detected following hybridization with a probe for DNA-PKcs (Hartley *et al.*, 1985, Cell **82**, 849-856). mRNA was extracted from untreated EcR293 clone 11 (track 1), and cells expressing NO following treatment with 1 μ M muristerone A (track 2, 1.2 ± 0.3 fold increase) and 10 μ M muristerone A (track 3, 2.4 ± 0.5 fold increase). The level of DNA-PKcs mRNA was increased significantly in cells expressing NO following treatment with muristerone A as compared with the untreated controls. This increase is reduced upon addition of the NOS inhibitor L-NIO (10 μ M muristerone A in the presence of 10 μ M L-NIO; 1.2 ± 0.1 fold increase) (Figure 4b). To control for differences in RNA loading, the intensity of the DNA-PKcs signal is expressed as a percentage of the β -actin signal (Figures 4a and 4b, bottom panels). In control experiments, treatment of the parental cell line EcR293 (Invitrogen) with 10 μ M muristerone A is unable to produce any change in the level of DNA-PKcs mRNA. Experiments using a shorter induction time (four hours and 12 hours) also fail to produce any significant increase in the mRNA level for DNA-PKcs (data not shown).

To determine whether the increase in of DNA-PKcs mRNA levels correspond to an increase in protein, DNA-PKcs protein levels were examined using western blotting (Figure 5a). For whole-cell extracts, 20 μ g of protein from untreated cells (track 1), and cells generating NO following treatment with 1 μ M muristerone A (track 2), 5 μ M muristerone A (track 3), 10 μ M muristerone A (track 4) and 10 μ M muristerone A in the presence of 10 μ M L-NIO (track 5) were loaded in each well. Following gel electrophoresis and blotting, DNA-PKcs specific bands are detectable with a DNA-PKcs specific polyclonal antibody (Ab-FLA), raised against the whole protein (Song *et al.*, 1996, EMBO J. **15**, 3238-3246) and chemiluminescence. The level of DNA-PKcs in cell lysates increases as the concentration of NO increases, correlating with the increase in the dose of muristerone A. The maximum increase in the level of DNA-PKcs is seen following treatment with 10 μ M muristerone A for 24

hr. To ensure that the differences observed in the levels of DNA-PKcs are not artefacts of the whole cell preparation, nuclear extracts were also analysed, confirming our previous results. Nuclear DNA-PKcs levels are markedly increased in cells generating NO following treatment with muristerone A (Figure 5b, lanes 2 and 3) as compared with untreated cells (lane 1).

DNA-PK activity measurements were carried out using a DNA-PK 'pulldown' peptide assay as described above (Finnie *et al.*, 1995, Proc. Natl. Acad. Sci. USA 93, 2442-2447). There is an increase of up to 1.8 fold and 3.5 fold in DNA-PK activity (Figure 6a) in extracts from cells generating NO following treatment with 1 μ M and 10 μ M muristerone A respectively as compared with untreated control cells. The increase of DNA-PKcs activity can be reduced by addition of the NOS inhibitor L-NIO.

Nuclear protein DNA-PK assays were carried out using the SignaTECT DNA- Dependent Protein Kinase Assay System (Promega) and supported the findings from the whole-cell assays. DNA-PK activity is increased by up to 2.5 fold and 3.5 fold in cells generating NO following treatment with either 1 μ M or 10 μ M muristerone A as compared with untreated controls (Figure 6b). Furthermore, treatment of cells with the NOS inhibitor, L-NIO (10 μ M) results in a reduction in the NO-mediated increase in DNA-PK activity.

NO-mediated increase in DNA-Pcs expression protects cells from X-ray irradiation, bleomycin, cisplatin, adriomycin and UV-C irradiation

We have shown that the addition of muristerone A to EcR293 clone-11 cells dramatically increases the concentration of NO within the cells, accompanied by an increase in DNA-PK enzyme activity. To test the functional significance of the NO-mediated increase in DNA-PKcs levels, we subjected cells to high doses of DNA-damaging agents, such as X-ray irradiation, bleomycin, cisplatin, adriamycin, UV-irradiation and the NO donor S-nitroso-N-acetyl-D,L-penicillamine (SNAP).

Cells were X-ray irradiated using a Pantak X-ray machine (320 Kv, 10mA) at a dose rate of 6 Gy min⁻¹. The fraction of cells that survived exposure to a range of X-ray doses from 3-9 Gy showed that EcR293 clone 11 cells, generating NO as a

result of muristerone A treatment were better protected from DNA damage than were untreated cells (see Figure 7). In control experiments, both parental (untransfected) EcR293 cells treated with muristerone A and EcR293 clone 11 cells in the absence of muristerone A showed similarly poor levels of protection.

5 In another series of experiments, following treatment of EcR293 clone-11 cells with 10 μ M muristerone A, cultures were exposed to various test solutions for 3 days: SNAP (500 μ g/ml), bleomycin (150 μ g/ml), adriamycin (50 μ g/ml) and cisplatin (12.5 μ g/ml). 72hr after treatment, adherent and non-adherent cells were pooled and viability assessed by trypan blue exclusion and lactate dehydrogenase determination (LDH) assay. Figure 8a shows that cells treated with 10 μ M muristerone A exhibit a significant resistance to all four DNA-damaging agents with a 4-fold increase in resistance to SNAP, a 3-fold increase in resistance to bleomycin, a 1.9-fold increase in resistance to adriamycin and a 4.6-fold increase in resistance to cisplatin as judged by the trypan blue exclusion assay. LDH determination assays gave similar results (data not shown).

15 EcR293 clone-11 cells were extremely sensitive to treatment with cisplatin. 12.5 μ g/ml causes massive apoptotic cell death within 24hr (see Figure 8b). The amount of cell death was dose dependent: 30 μ g/ml causes 90% cell death; and 150 μ g/ml kills over 95% of cells in 24hr.

20 Remarkably, the NO-induced cells have over 85% protection against such toxicity even at the highest concentration (150 μ g/ml) of cisplatin. To confirm the role of DNA-PK in this protection, 20 μ M wortmannin (a specific inhibitor of PI-3-type kinases) was added in the 10 μ M muristerone A induced culture medium. Wortmannin (WM) completely abolished the protective role of NO-generation against the damaging effect of cisplatin (see Figure 8b).

25 We have also tested the cells for UV-C sensitivity. The exposure of control EcR293 clone-11 cells to UV-C irradiation results in a dose-dependent increase in cell death. More than half the resting cells are dead at doses of 30mJ/cm² as judged by the trypan blue exclusion assay. Exposure of cells to UV-C irradiation (120mJ/cm², for 24hr) results in 95% cell killing (see Figure 8c).

30 Remarkably, nearly 90% protection against UV-C induced cell death is

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observed following treatment with 10 μ M muristerone A (see Figure 8d) as judged by examination of the cells with TdT (terminal deoxynucleotidyl transferase) mediated dUTP-biotin nick end labeling (TUNEL) method. This dose of muristerone A results in a 3 to 5-fold increase in DNA-PKcs activity as compared with untreated control cells. Furthermore, addition of the NOS inhibitor, L-NIO (20 μ M) decreases DNA-PK activity and abrogates the protective role of NO generation against UV-C irradiation (see Figure 8d). 20 μ M wortmannin treatment alone has no toxic effect under the same culture conditions (see Figure 8d, WM/-UV column). In each sample, a minimum of 500 cells were counted and labeled nuclei were expressed as a percentage of the total number of nuclei. Values are the means \pm S.D. of 3 to 5 individual experiments. **, P<0.01.

Discussion

We report the finding that NO up-regulates the transcription of the catalytic subunit of DNA-PK, correlating with an increase in enzyme activity for DNA-PK. This is a novel finding, as previous reports have demonstrated DNA-PK levels do not fluctuate in a cell (Lee *et al.*, 1997, *Molec. Cell. Bio.* 17, 1425-1433). Furthermore, even very high doses of ionizing radiation do not result in any significant changes in protein levels or activity for DNA-PK in either human or rodent cells (Lees-Miller *et al.*, 1995, *Science* 267, 1183-1185). The observation that NO can mediate an increase in DNA-PKcs transcriptional activity correlating with an increase in enzyme activity, points to a new mechanism for DNA-PK regulation.

The NO mediated increase in DNA-PK activity is likely to have significant biological consequences. DNA-PK is important in DNA repair, and SCID mice with a DNA-PKcs deficiency have an increased susceptibility to ionizing radiation, impaired V(D)J recombination and arrested B and T cell development (Siple *et al.*, 1995, *Proc. Natl. Acad. Sci. USA* 92, 7515-7519; Miller *et al.*, 1995, *Proc. Natl. Acad. Sci. USA* 92, 10792-10795). Furthermore, DNA-PKcs mutant cells (scid) show increased sensitivity to UV-C irradiation (2 to 2.5 fold) and cisplatin (3 to 4 fold). Recent studies on retroviral DNA integration has shown that DNA-PKcs is also involved in the process of retroviral integration (Daniel *et al.*, 1998, *Science*

284(5414), 644-7). It has been shown that retroviral integration, for HIV and other retroviruses, requires the activity of the DNA-PKcs mediated pathway. Therefore, a DNA-PKcs inhibitor or an inhibitor of NOS may be useful in the treatment of retrovirus, for example HIV, infection.

5 Although NO mediates an increase in both mRNA and protein for DNA-PKcs, increased enzyme activity requires associated DNA damage within the cell. It is possible that the NO-mediated increase in both mRNA and protein for DNA-PKcs acts as a "priming mechanism" enabling the cell to respond rapidly to NO-associated DNA damage. Previous reports have shown that NO triggers DNA damage (Wink *et al.*, 1991, Science **254**, 1001-1003; Nguyen *et al.*, 1992, Proc. Natl. Acad. Sci. USA **89**, 3030-3034) and, that this in turn, activated poly (ADP-ribose) polymerase (PARP), a DNA break activated molecule involved in genomic stability (Le Rhun *et al.*, 1998, Biochem. Biophys. Res. Commun. **245**, 1-10; Zhang *et al.*, 1994, Science **263**, 687-689). The NO-mediated increase in activity of both DNA-PK and PARP suggests there may be a co-ordinated response within the cell to minimise potentially genotoxic mediated by NO.

10 Exposure of cells to low doses of NO has been shown to offer protection against subsequent challenge with much higher doses (Kim *et al.*, 1995, FEBS Lett. **374**, 228-232). More recently, NO has been demonstrated to protect keratinocytes and endothelial cells against UVA-induced DNA damage and apoptosis by increasing Bcl-expression (Suschek *et al.*, 1999, J. Biol. Chem. **274**, 6130-6137). These findings, taken together with the results presented here supports the idea of NO having a signal "priming role", enabling the cell to respond rapidly to subsequent NO and radiation-associated damage.

20 To further test the biological significance of the upregulation of DNA-PK we exposed cells to high doses of DNA damaging agents, such as NO donors, bleomycin, adriamycin, cisplatin, UV-C irradiation and X-ray irradiation. Some of these agents, such as bleomycin and cisplatin, are highly efficient anticancer drugs which work via mechanisms resulting in DNA damage, including both single and double-strand damage. We have shown that NO generating cells, with increased levels of DNA-PK, are fully protected against UV-C, X-ray irradiation, bleomycin,

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adriamycin and cisplatin, as well as to high concentrations of NO donors. However, our data show that a NOS inhibitor or a DNA-PK inhibitor can abolish this protection.

These results are highly significant in the context of cancer therapy. The finding that human cancer cells express NOS indicates that NO may play a pathophysiological role in promoting tumor growth and in protecting tumors from agents that cause DNA damage. NO production in cancer cells may thus confer resistance to chemotherapeutic drugs, such as bleomycin and cisplatin, and radiotherapeutic agents, such as X-ray irradiation, on those cells.

10 Acquired drug resistance is a major problem in cancer treatment. Our findings suggest that NO production may underlie resistance to some widely used cancer drugs. These findings open up a totally new strategy for cancer therapy, suggesting that administration of DNA-damaging drugs in combination with inhibitors of NOS or DNA-PK can sensitize NO-producing tumor cells that would
15 otherwise be resistant to DNA-damaging drugs or agents.

Claims

1. A method for identifying a polynucleotide, the expression of which is modulated in the presence of nitric oxide (NO), which method comprises:

- 5 (i) providing an mRNA or cDNA population from cells which contain a polynucleotide construct, which construct comprises:
- (a) a promoter operably linked to a coding sequence, wherein the promoter is responsive to ecdysone or an analog thereof and the coding sequence encodes a nitric oxide synthase (NOS) or
- 10 a functional variant thereof; or
- (b) a promoter operably linked to one or more tetracycline operator site sequences and a coding sequence in that order, wherein the coding sequence encodes a nitric oxide synthase (NOS) or a functional variant thereof;
- 15 (ii) providing an mRNA or cDNA population from cells as defined in step (i), said cells having been contacted with ecdysone or an analog thereof; and
- (iii) comparing the populations of steps (i) and (ii), thereby to determine which polynucleotides show modulated expression in the presence of
- 20 NO.

2. Use of a polynucleotide identified by a method according to claim 1 in a method for identifying an inhibitor or stimulator of transcription and/or translation of the polynucleotide and/or activity of the polypeptide encoded by that

25 polynucleotide.

3. A method for identifying:

- (i) an inhibitor or stimulator of transcription and/or translation of a polynucleotide identified by a method according to claim 1; and/or
- 30 (ii) an inhibitor or stimulator of activity of a polypeptide encoded

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by a said polynucleotide,
which method comprises determining whether a test substance can inhibit or stimulate transcription and/or translation of the polynucleotide and/or activity of a polypeptide encoded by a said polynucleotide.

4. An inhibitor or stimulator identified by the method of claim 3.

5. An inhibitor or stimulator according to claim 4 for use in a method of treatment of the human or animal body by therapy.

6. A polynucleotide construct comprising:

(a) a promoter operably linked to a coding sequence, wherein the promoter is responsive to ecdysone or an analog thereof and the coding sequence encodes a nitric oxide synthase (NOS) or a functional variant thereof; or

(b) a promoter operably linked to one or more tetracycline operator site sequences and a coding sequence in that order, wherein the coding sequence encodes a nitric oxide synthase (NOS) or a functional variant thereof.

7. A polynucleotide construct according to claim 6, wherein the NOS is human inducible NOS.

8. A polynucleotide construct according to claim 6, wherein the NOS is human neuronal NOS.

9. A polynucleotide construct according to claim 6, wherein the NOS is human endothelial NOS.

10. A polynucleotide construct according to any one of claims 6 to 9, wherein the promoter in part (a) comprises a minimal promoter and an element or

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elements which is/are responsive to ecdysone or an analog thereof.

11. A polynucleotide construct according to any one of claims 6 to 9, wherein two operator site sequences are present in part (b).

12. A vector which incorporates a polynucleotide construct as defined in any one of claims 6 to 11.

10 13. A cell which harbours a polynucleotide construct according to any one of claims 6 to 11 or a vector according to claim 12.

14. A cell according to claim 13 which harbours a construct as defined in part (a) of claim 6 and which is capable of expressing a functional ecdysone receptor.

15 15. A cell according to claim 14, wherein the functional ecdysone receptor comprises a heterodimer of the ecdysone receptor (EcR) or functional variant thereof and the human retinoid X receptor (RXR) or functional variant thereof.

20 16. A cell according to claim 13 which harbours a construct as defined in part (b) of claim 6 and which is capable of expressing the tetracycline repressor protein or a functional variant thereof.

25 17. Products containing an NOS inhibitor and a DNA damaging agent as a combined preparation for simultaneous, separate or sequential use in the treatment of cancer.

30 18. Products containing a DNA repair enzyme inhibitor and a DNA damaging agent as a combined preparation for simultaneous, separate or sequential use in the treatment of cancer.

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19. Products according to claim 18, wherein the DNA repair enzyme inhibitor is a PI 3-kinase like kinase inhibitor.

20. Products according to claim 19, wherein the PI 3-kinase like kinase inhibitor is a DNA-PK inhibitor.

21. Products according to any one of claims 17 to 19, wherein the DNA damaging agent is a DNA alkylating or cross-linking agent.

22. Products according to claim 21, wherein the alkylating or cross-linking agent is a nitrosourea, a nitrogen mustard, a mitomycin or a platinum coordination compound.

23. Products according to any one of claims 17 to 19, wherein the DNA damaging agent is a DNA binding and/or cleaving agent.

24. Products according to claim 23, wherein the DNA binding and/or cleaving agent is danorubicin hydrochloride USP (Cerubidine), doxorubicin USP (Adriamycin), idarubicin hydrochloride (Idamycin), mitoxanthrone hydrochloride USP (Novantrone), bleomycin sulfate USP (Blenoxane), esperamicin A₁, dactinomycin USP (Cosmegen), plicamycin USP, procarbazine hydrochloride USP (Matulane).

25. Use of an NOS inhibitor in the manufacture of a medicament for use with a DNA damaging agent in the treatment of cancer.

26. Use of a DNA repair enzyme inhibitor in the manufacture of a medicament for use with a DNA damaging agent in the treatment of cancer.

27. Use according to claim 26, wherein the DNA repair enzyme is a PI 3-kinase like kinase inhibitor.

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28. Use according to claim 27, wherein the PI 3-kinase like kinase inhibitor is a DNA-PK inhibitor.

29. Use according to any one of claims 25 to 28, wherein the DNA
5 damaging agent is a DNA alkylating and/or cross-linking agent.

30. Use according to claim 29, wherein the DNA alkylating or cross-linking agent is a nitrosourea, a nitrogen mustard, a mitomycin or a platinum coordination compound.

10 31. Use according to any one of claims 25 or 28, wherein the DNA damaging agent is a DNA binding and/or cleaving agent.

15 32. Use according to claim 31, wherein the DNA binding and/or cleaving agent is danorubicin hydrochloride USP (Cerubidine), doxorubicin USP (Adriamycin), idarubicin hydrochloride (Idamycin), mitoxanthrone hydrochloride USP (Novantrone), bleomycin sulfate USP (Blenoxane), dactinomycin USP (Cosmegen), plicamycin USP, procarbazine hydrochloride USP (Matulane).

20 33. Use according to any one of claims 25 to 28, wherein the DNA damaging agent is X-ray irradiation.

25 34. A method of treating a host suffering from a cancer, which method comprises administering to the host therapeutically effective amounts of an NOS inhibitor and a DNA damaging agent.

30 35. A method of treating a host suffering from a cancer, which method comprises administering to the host therapeutically effective amounts of a DNA repair enzyme inhibitor and a DNA damaging agent.

36. A method according to claim 35, wherein the DNA repair enzyme is a

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PI 3-kinase like kinase inhibitor.

37. A method according to claim 36, wherein the PI 3-kinase like kinase inhibitor is a DNA-PK inhibitor.

38. A method according to any one of claims 34 to 37, wherein the DNA damaging agent is a DNA alkylating and/or cross-linking agent.

39. A method according to claim 38, wherein the DNA alkylating and/or cross-linking agent is a nitrosourea, a nitrogen mustard, a mitomycin or a platinum coordination compound.

40. A method according to any one of claims 34 to 37, wherein the DNA damaging agent is a DNA binding and/or cleaving agent.

41. A method according to claim 40, wherein the DNA binding and/or cleaving agent is danorubicin hydrochloride USP (Cerubidine), doxorubicin USP (Adriamycin), idarubicin hydrochloride (Idamycin), mitoxanthrone hydrochloride USP (Novantrone), bleomycin sulfate USP (Blenoxane), dactinomycin USP (Cosmegen), plicamycin USP, procarbazine hydrochloride USP (Matulane).

42. A method according to any one of claims 34 to 37, wherein the DNA damaging agent is X-ray irradiation.

43. Use of an NOS inhibitor in the manufacture of a medicament for use in the treatment of retroviral infection.

44. Use of an NOS inhibitor in the manufacture of a medicament for use with a PI 3-kinase like kinase inhibitor in the treatment of retroviral infection.

45. Use according to claim 44, wherein the PI 3-kinase like kinase is

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DNA-PKcs.

46. Products containing an NOS inhibitor and a PI 3-kinase like kinase inhibitor as a combined preparation for simultaneous, separate or sequential use in the treatment of retroviral infection.

47. Products according to claim 46, wherein the PI 3-kinase like kinase is DNA-PKcs.

48. A method of treating a host suffering from retroviral infection, which method comprises administering to the host a therapeutically effective amount of an NOS inhibitor.

49. A method of treating a host suffering from retroviral infection, which method comprises administering to the host therapeutically effective amounts of an NOS inhibitor and a PI-3 kinase like kinase inhibitor.

50. A method according to claim 49, wherein the PI 3-kinase like kinase is DNA-PKcs.

51. Use according to any one of claims 43 to 45, products according to claim 46 or 47 or a method according to any one of claims 48 to 50, wherein the retroviral infection is HIV infection.

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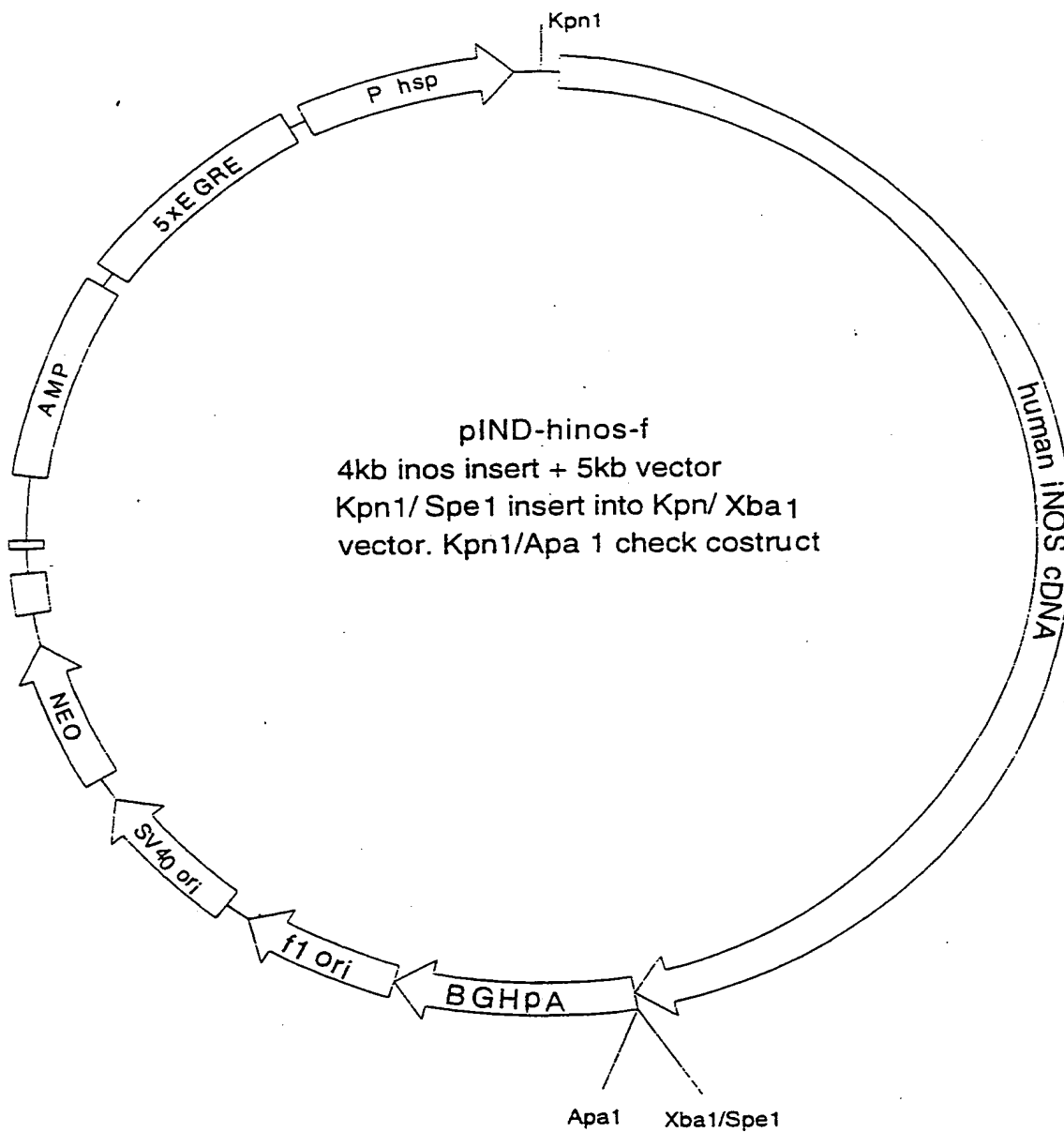


Figure 1a

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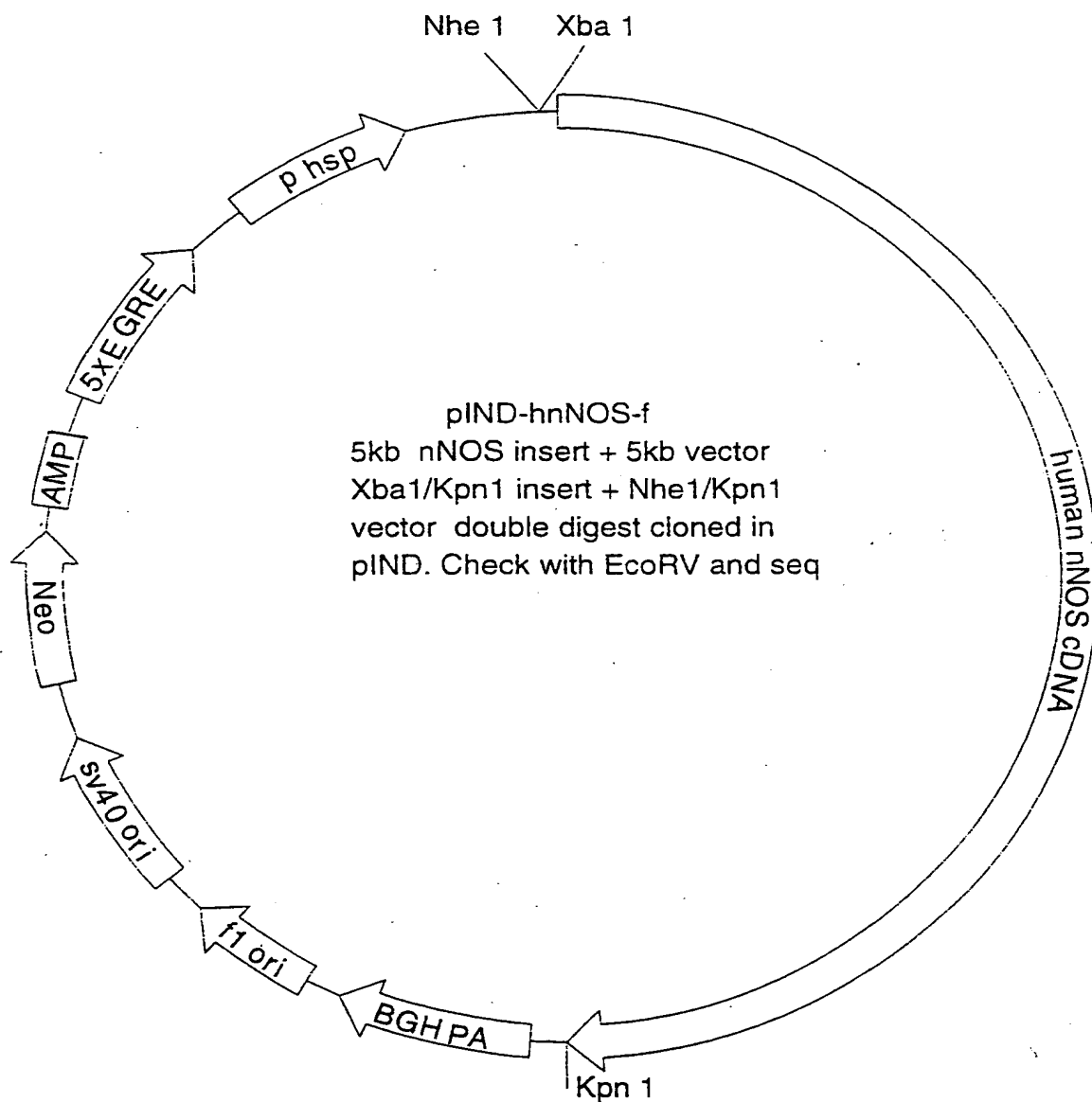


Figure 1b

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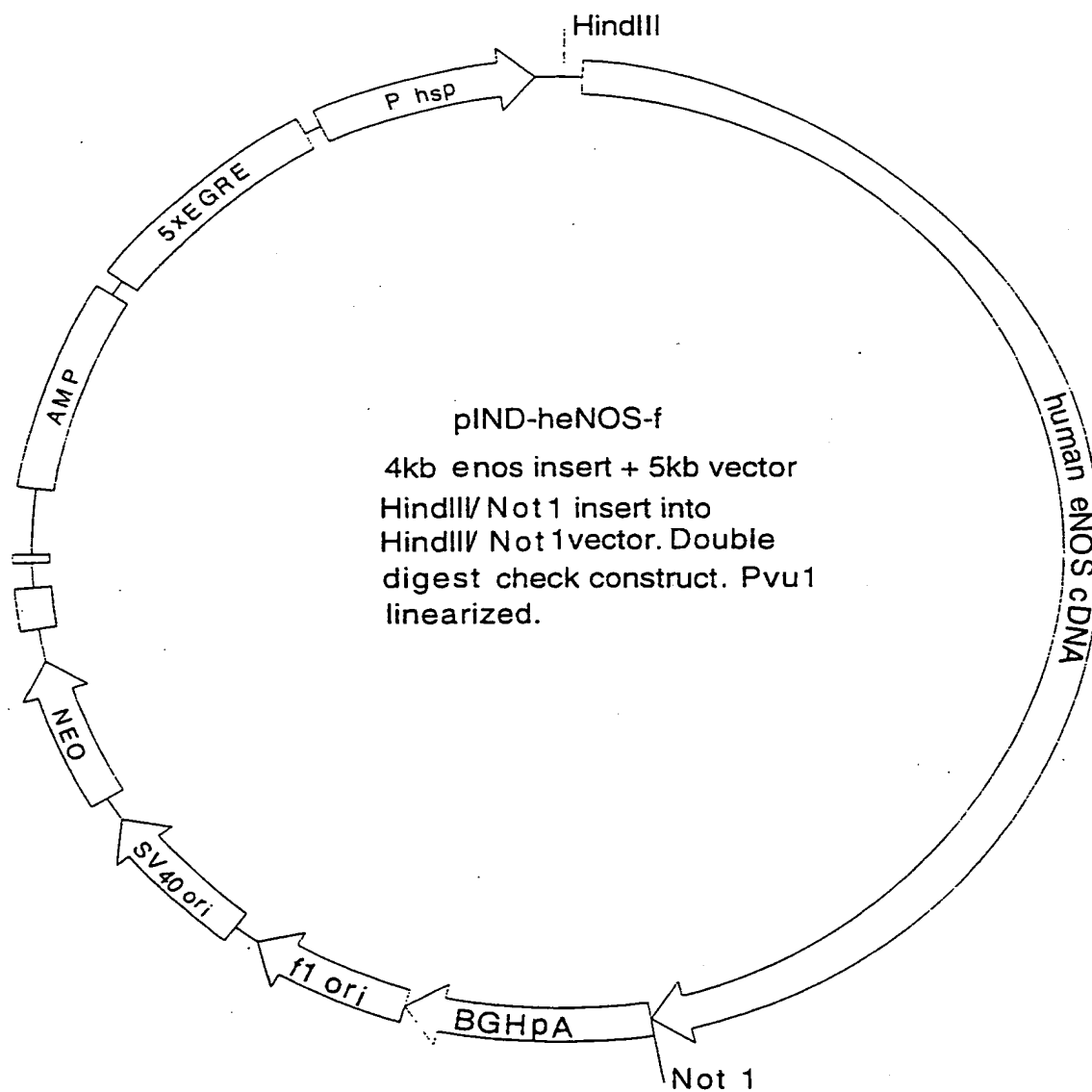


Figure 1c

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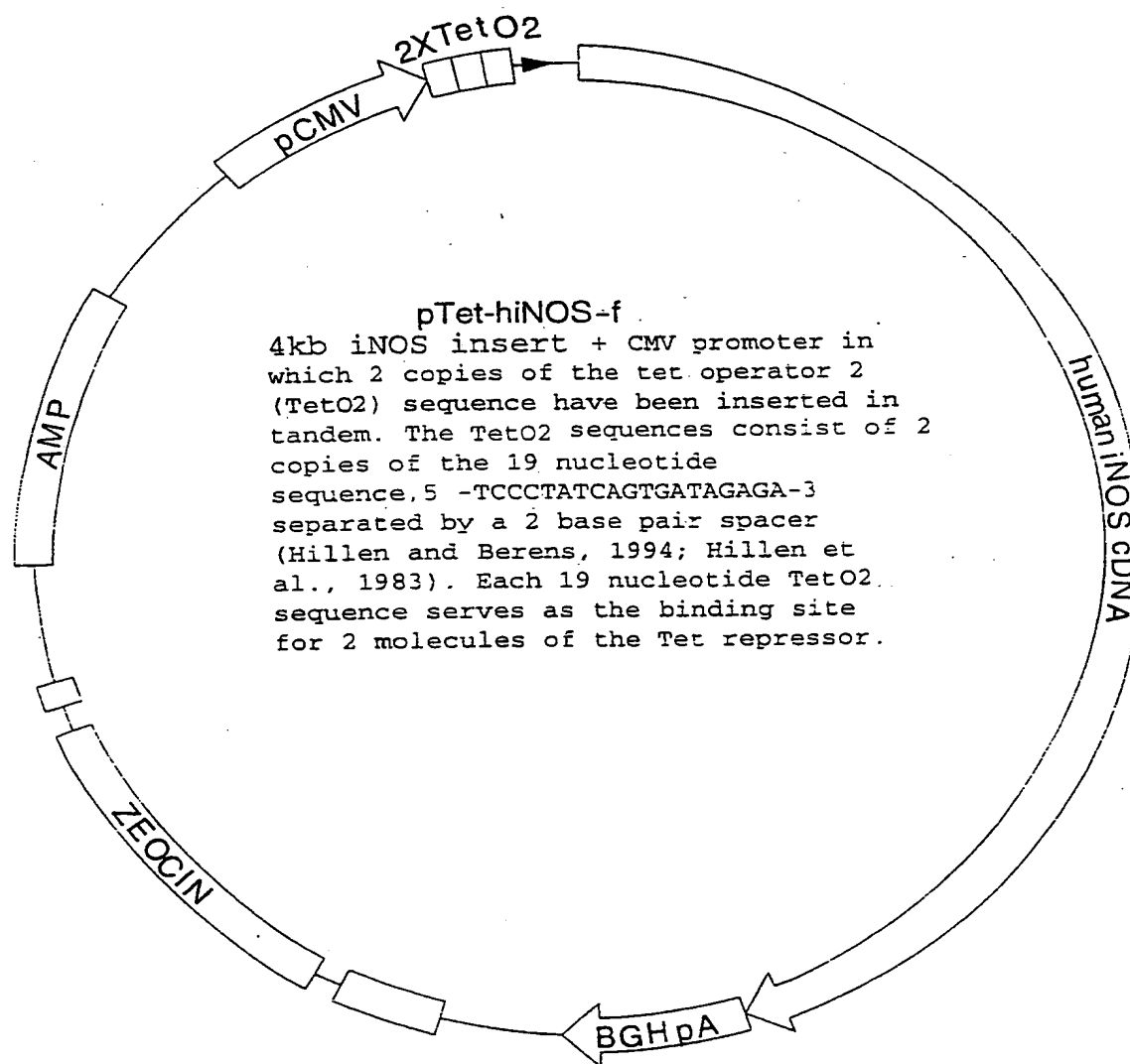


Figure 1d

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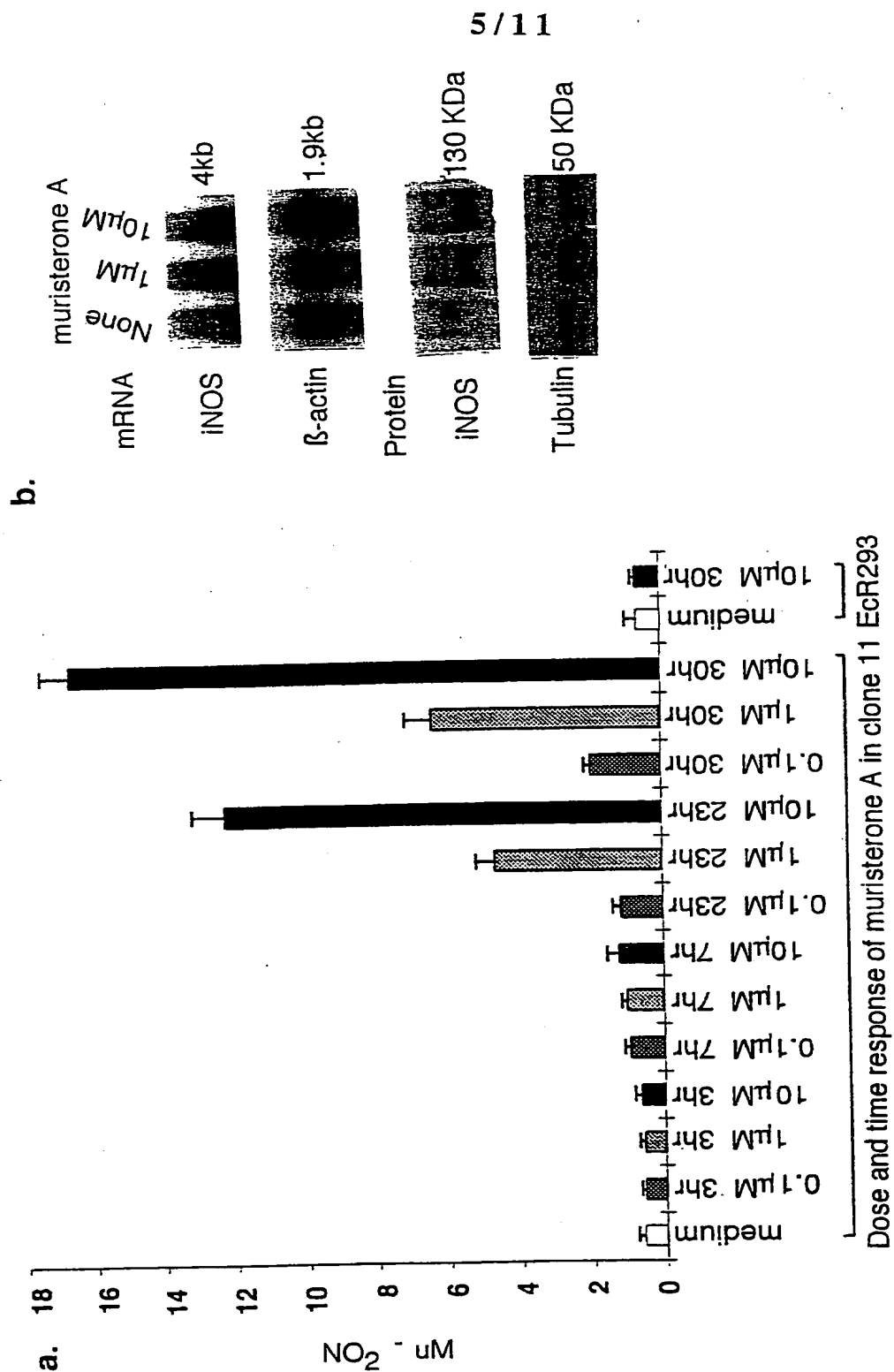
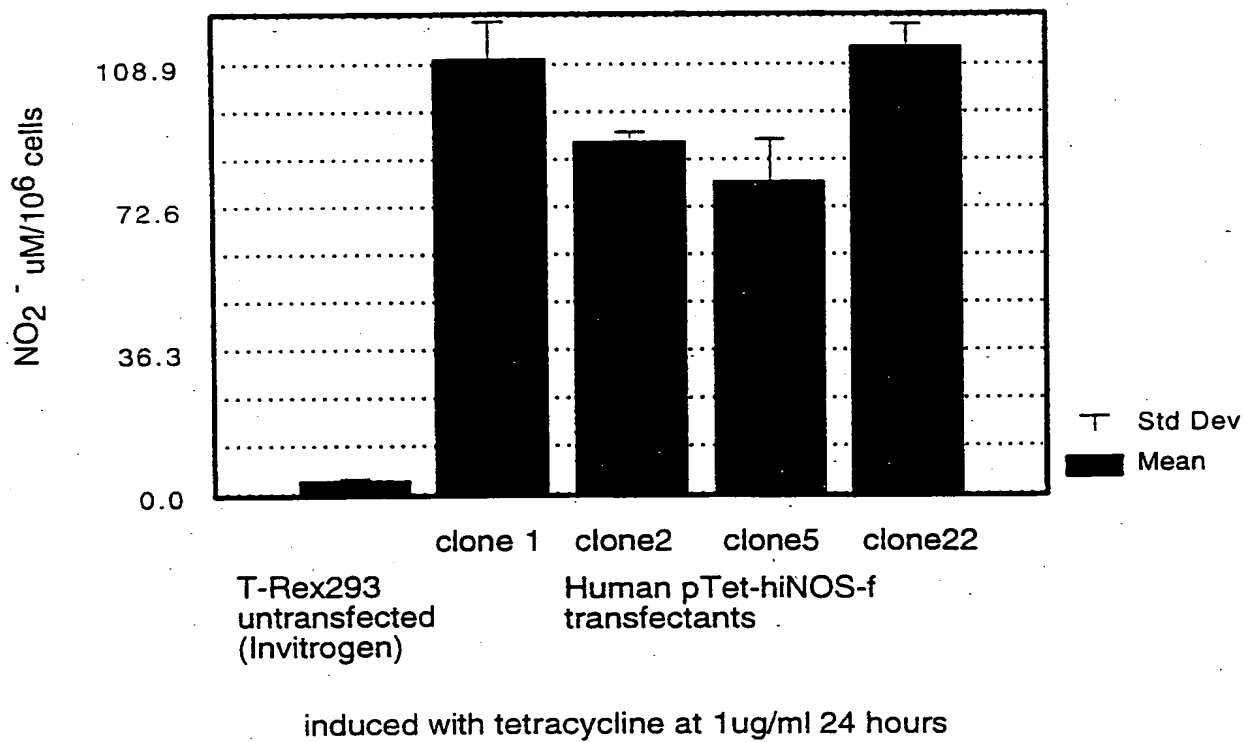


Figure 2

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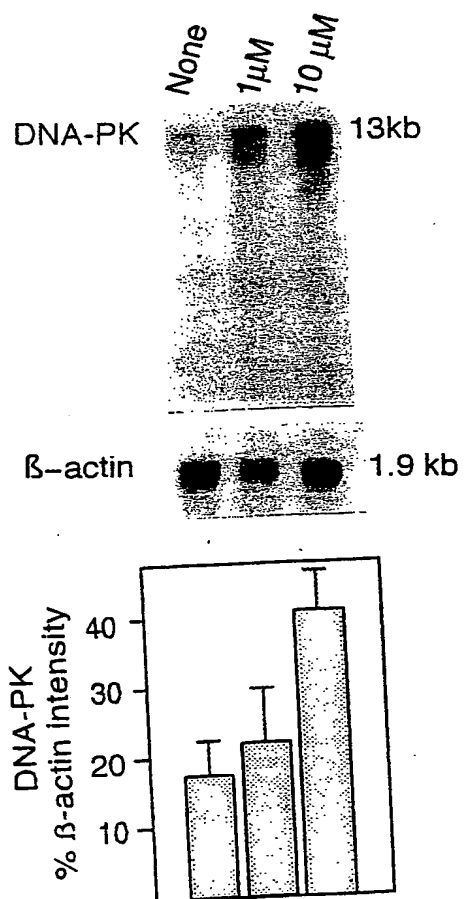
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**Figure 3**

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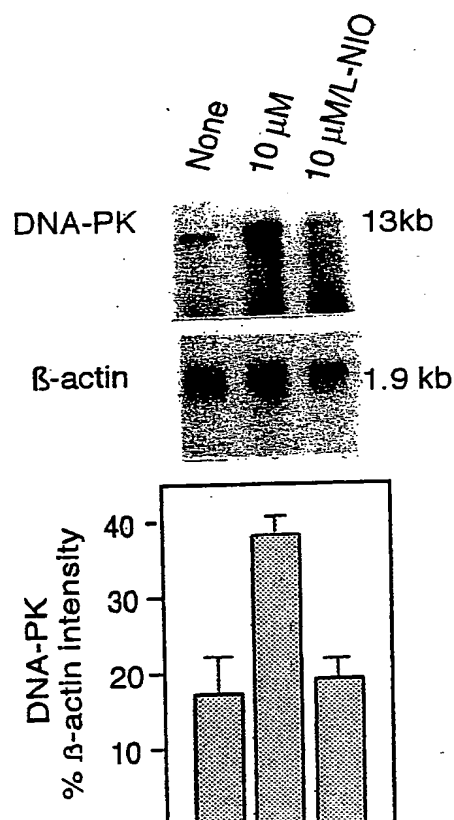
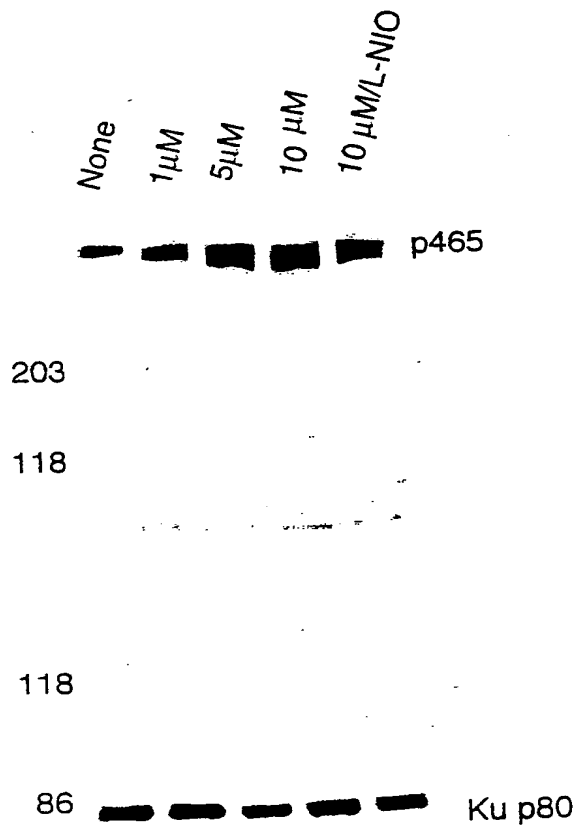


Figure 4

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a.



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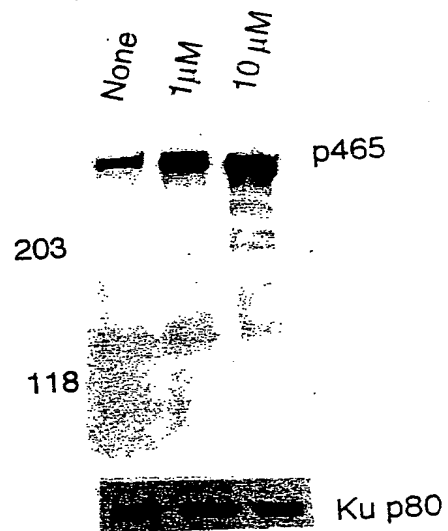


Figure 5

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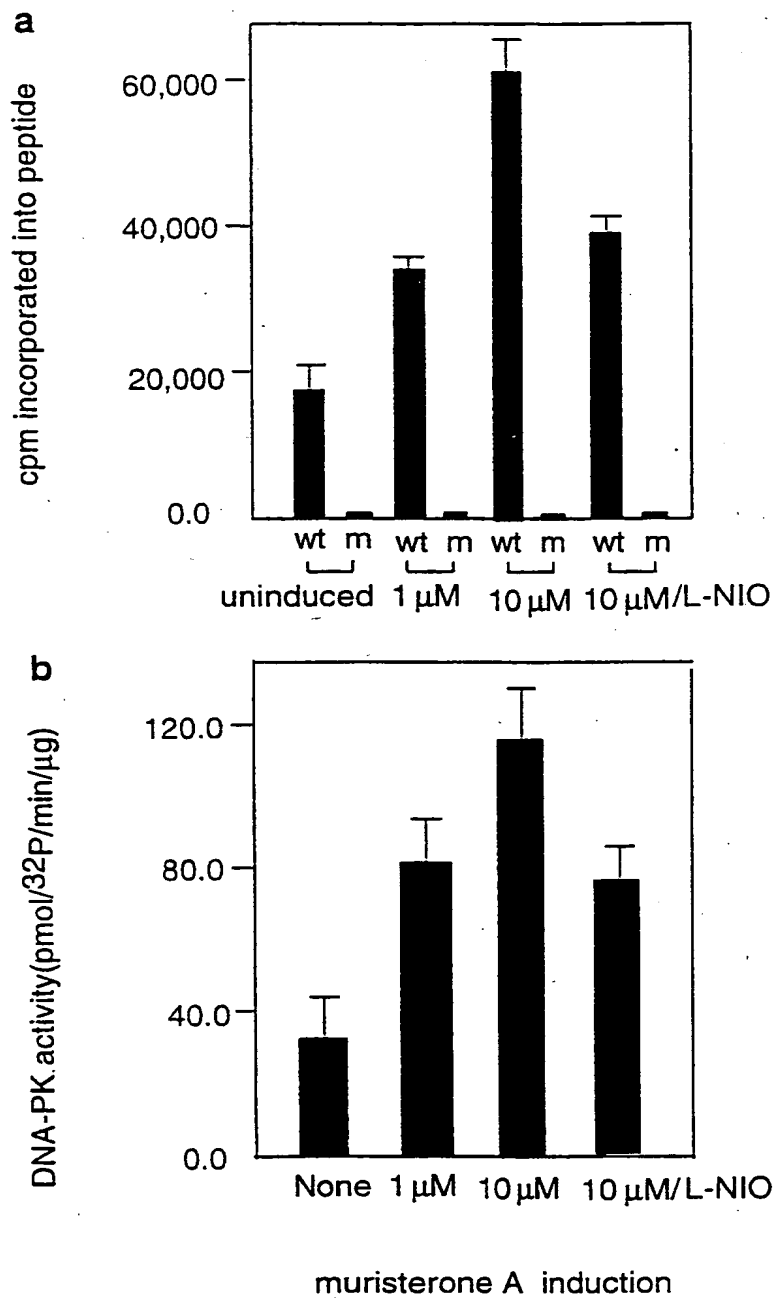


Figure 6

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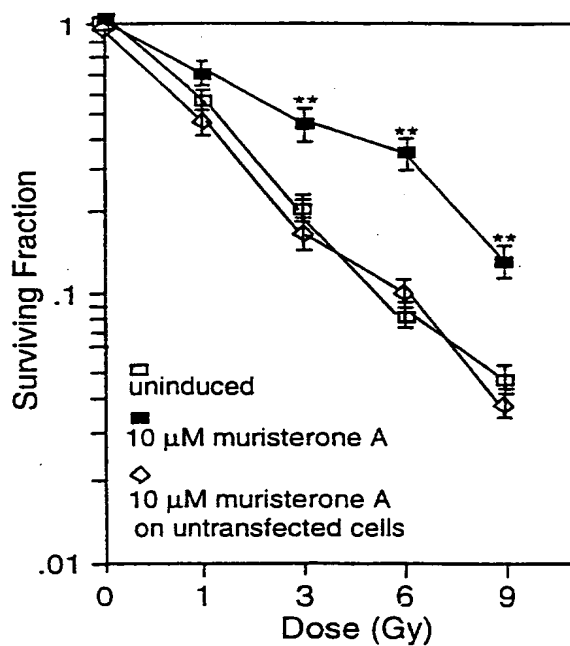


Figure 7

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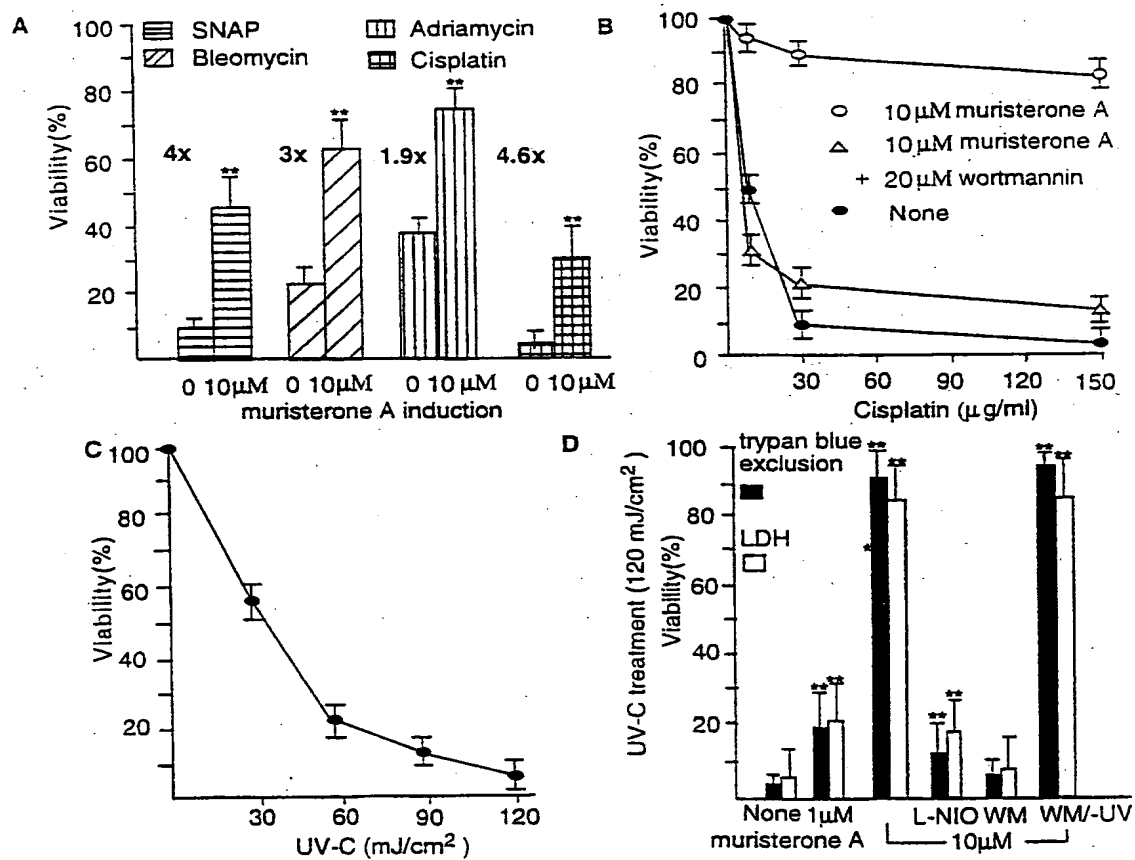


Figure 8

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